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The Fractionation and Enzymic Breakdown of some Phosphorus Compounds in Leaf Tissue

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(*Received* 28 *September* 1951)

In a recent paper Pirie (1950) has described the preparation and properties of a nucleoprotein, from the sap of healthy tobacco leaves, which has some similarity to plant viruses. The present paper is concerned with the fractionation of the phosphorus of leaf tissue to find how much of the phosphorus of the leaf is in the form of nucleic acid. The enzymic breakdown of some of the phosphorus-containing compounds has also been studied.

Although there is an extensive literature on the nature and fractionation of phosphorus compounds in animal tissues, plant tissues have received less attention. Until recently, work on plant tissues has been mainly confined to seeds. When leaves have been used the material has usually been dried as a preliminary. This will vitiate some of the results, for there would be changes due to enzyme action during drying, especially at room temperature. Knowles & Watkin (1932) investigated the phosphorus compounds in wheat at different stages of growth. They used air-dry material, which probably accounts for the very high figures for inorganic phosphorus. They claim to have found phytin in vegetative parts of the wheat plant, but other workers have been unable to find any phytin except in seeds. DeTurk, Holbert & Howk (1933) studied the chemical transformations of phosphorus in the growing corn plant and found that phytin disappeared rapidly on germination. They found differences in the phosphorus distribution between fractions when the material was slowly and rapidly dried.

Heard (1945) investigated the forms of phosphorus in a trichloroacetic acid extract of fresh barley seedlings. In a study of the nucleic acid content of green leaves, von Euler & Hahn (1947, 1948) extracted ribonucleic acid with sodium hydroxide as in the Schmidt & Thannhauser (1945) procedure for animal tissues. The nucleic acid was precipitated as the lanthanum salt and the pentose content determined by a phloroglucinol method. Pectic substances in the extract were precipitated with the nucleic acid, and estimation of the nucleic acid content based on the pentose figure gave values which were too high. Williams (1945) described a method for estimating nucleic acid phosphorus in plant material in which the dry fat-free tissue was heated with 12·5% (w/v) sodium chloride in the presence of triacetin. The nucleic acid in the extract was then precipitated at pH 1. In this method no distinction was made between ribo- and deoxyribonucleic acids. The method of Ogur & Rosen (1950) which, with modifications, was used in the present investigation for the differential extraction of ribo- and deoxyribo-nucleic acids was used by them on fresh corn root tips.

The enzymes in plant tissues concerned with the breakdown of phosphorus compounds have also been studied much less frequently than those of animal tissues. The presence of phosphatase activity in leaves is well known. Courtois & Khorsand (1950) have recently found that there are two phosphatases, one with an optimum pH at 5·0–5·2 and the other at about pH 4. Schlamowitz & Garner (1946) recognized a ribonuclease ('ribonucleinase') in sprouted soy beans. Pirie (1950) found that tobacco-leaf sap had ribonuclease activity. Hanahan & Chaikoff (1948) found a phospholipin-splitting enzyme in cabbage leaves which was able to attack only the nitrogenous base-phosphoric acid linkage of lecithin with the formation of choline and phosphatidic acid. Ducet (1949) found this enzyme and also lecithinase *B*, which splits lecithin to glycerophosphorylcholine and fatty acids, in numerous other plants such as potato, bean, pea and ryegrass.

MATERIAL AND METHODS

Leaves from glasshouse-grown tobacco plants (*Nicotiana tabacum* var. White Burley) were used for most of the work. Other species used are mentioned in the text. The midribs were cut out, the laminae minced in a domestic meat mincer and the sap squeezed out by hand through madapollam into a cooled receiver. The residue in the cloth, called 'fibre', was washed three times by suspending in a volume of distilled water about equal to that of the sap and squeezing out. If not used at once the fibre was stored at 4° with $CHCl_3$ added. The sap was centrifuged at 8000 rev./min. (6000*g*) for 15 min. and the deposit obtained called the 'chloroplast fraction'. This was washed twice by suspending in distilled water and recentrifuging at 8000 rev./min., then it was finally suspended in water and stored in the refrigerator.

For some experiments much of the starch was removed from the chloroplast fraction by scraping off the upper green layer from the white starch layer in the deposit obtained on centrifuging. The green material was suspended in water, centrifuged down again and separated from the starch.

This was repeated until there was no obvious starch layer.

Phosphorus. This was determined colorimetrically by a modification of the method of Kuttner & Lichtenstein (1932). Values for inorganic P were obtained by developing the colour in samples without incineration.

Dry matter of fibre and of aqueous fractions. This was determined by drying samples in an oven at 100° overnight. Ethanol-ether fractions were dried over H_2SO_4 in a vacuum desiccator.

Nitrogen. Total N was determined by a micro-Kjeldahl method using SeO_2:$CuSO_4$:K_2SO_4 (1:1:8) catalyst.

Carbohydrate. Total carbohydrate was determined by the orcinol method (Pirie, 1936), reducing sugar by the Hanes (1929) modification of the Hagedorn & Jensen method and uronic acid by the method of Tracey (1948).

Deoxyribonucleic acid (DNA). This was determined by the diphenylamine method (Dische, 1930) and the cysteine-sulphuric acid method (Stumpf, 1947), and a modification of the tryptophan-perchloric acid method of Cohen (1944).

Determination of phosphatase activity. The liberation of inorganic P from sodium β-glycerophosphate was used to measure phosphatase activity. For the tests a total volume of 5 ml. contained sodium β-glycerophosphate solution (pH 6) to give 100 μg. P/ml., sodium citrate buffer (pH 6) 0·04 M, enzyme solution and water to make up the volume. Samples were removed at intervals and pipetted into 5 ml. of 2 N-H_2SO_4 and inorganic P determined.

Determination of nuclease activity. The liberation of P not precipitable by uranyl nitrate in trichloroacetic acid (TCA) (MacFadyen, 1934) from yeast nucleic acid was used to measure ribonuclease activity. For the tests a total volume of 5 ml. contained sufficient of the sodium salt of yeast ribonucleic acid to give 100 μg. P/ml., sodium citrate buffer 0·04 M (pH 6), enzyme solution and water to make up the volume. Samples were removed at intervals, made up to 1 ml., and 1 ml. uranyl nitrate solution 0·5 % (w/v) in 2·5 % (w/v) TCA was added. After standing the tubes on ice for 10 min. the precipitates were centrifuged down and total P determined in the supernatants. Deoxyribonuclease activity was determined similarly, using a solution of the sodium salt of deoxyribonucleic acid from thymus (British Drug Houses Ltd.) and either sodium diethylbarbiturate-sodium acetate buffer (pH 7), or an α-picoline-acetic acid buffer (pH 6) (Cecil, 1950).

Ultraviolet absorption spectra. These were obtained with a Hilger absorption spectrograph and with a Unicam quartz spectrophotometer.

pH *measurements.* These were made with a glass electrode.

RESULTS

Fractionation of phosphorus compounds in fibre, chloroplast and some sap fractions

Ogur & Rosen (1950) have recently described a method of fractionating P compounds in plant tissue involving the use of perchloric acid. This method has been modified, and forms the basis of the fractionation procedure used in the work described here.

Fibre

The P compounds of tobacco-leaf fibre have been fractionated into four groups: (1) P soluble in cold dilute acid; (2) P soluble in ethanol-ether; (3) P soluble in N-$HClO_4$ on soaking overnight at room temperature; (4) P soluble in N-$HClO_4$ on incubating at 37° overnight. The extraction of these removed over 98 % of the fibre P. Table 1 shows the results of a typical experiment.

Table 1. *Fractionation of phosphorus in fresh and incubated fibre*

(3 g. lots of fibre (dry matter 0·617 g.) extracted successively as shown in the table. The method is described in the text, p. 434. The water extract from the incubated fibre was obtained by incubating the fibre twice in water at 37° overnight.)

	P in fresh fibre		P in incubated fibre	
Extractant	(mg./g. of dry fibre)	(% of total in fibre)	(mg./g. of dry fibre)	(% of total in fibre)
1. Water, 30 ml.	0·097	3·3	2·356	76·1
2. 0·2 N-$HClO_4$, 30 ml.	0·102	3·5	0·073	2·4
3. Ethanol-ether (3:1), 40 ml., i.e. lipid	0·64	21·8	0·082	2·6
4. N-$HClO_4$ at 16° overnight, 30 ml., i.e. RNAP*	1·61	54·8	0·194	6·3
5. Two extractions with N-$HClO_4$ at 37° overnight, 40 ml., i.e. mainly DNAP†	0·485	16·5	0·391	12·6
Totals	2·934	—	3·096	—

* RNAP, ribonucleic acid phosphorus.

† DNAP, deoxyribonucleic acid phosphorus.

As soon as possible after mincing and washing, two 3 g. lots of fibre were suspended in 20 ml. water. One lot was incubated twice, each time for about 24 hr. at 37° with $CHCl_3$ present. The other was squeezed out at once and re-washed with 10 ml. water. The fibre was then extracted with two lots of 0·2 N-$HClO_4$, 20 ml. and 10 ml., which were combined. The acid was not left in contact with the fibre for longer than 10 min. at room temperature. The fibre was then extracted with ethanol-ether (3:1) at room temperature until no more colour could be removed, as the removal of P paralleled the removal of pigments. The fibre was then suspended in 20 ml. N-$HClO_4$ and kept at room temperature overnight, i.e. about 16° for 18 hr. After removal of the extract the fibre was washed with 10 ml. N-$HClO_4$ and the N-$HClO_4$ extracts pooled. The fibre was then suspended in 20 ml. N-$HClO_4$ and incubated at 37° overnight, washed with 10 ml. N-$HClO_4$ and the extracts pooled. The extract from a second incubation at 37° overnight with 10 ml. N-$HClO_4$ was added to the first incubation extract.

The portion which was incubated at 37° in water was afterwards extracted with ethanol-ether and $HClO_4$ in exactly the same way as the other portion.

(1) *Cold dilute acid-soluble* P. For the extraction of this fraction 0·2 N-HCl, 0·2 N-$HClO_4$ and 5% (w/v) TCA were used and the results compared. There was no significant difference in the amount of P extracted by these acids. This fraction amounted to not more than 10% of the total P in well washed fresh fibre. From 30 to 50% of the P in this fraction was not in the form of PO_4^{---} and its nature has not been investigated. In fibre that had been stored in the refrigerator for 2–3 weeks as much as 30% of the total P of the fibre was in the acid-soluble fraction. The increased amount extractable was inorganic P due to enzymic breakdown of P compounds and could be removed with water alone, without acid.

(The small leaves were less than 10 cm. long and the large from 10 to 25 cm. long.) This shows that a much smaller proportion of the N was lost on incubation than of the P. The phospholipid-splitting enzyme investigated by Hanahan & Chaikoff (1948) caused only 5% loss of ether-soluble P compared with 35% loss of N when soy bean phospholipin was used as substrate. In the present investigation when cabbage-leaf fibre was incubated in water at 37° it behaved similarly to tobacco with the ethanol-ether soluble P being much reduced.

(3) *and* (4) P *soluble in* N-$HClO_4$. When fibre which had been washed with cold 5% (w/v) TCA was heated at 90° for 15 min. in 5% TCA (Schneider, 1945) or incubated at 37° for

Table 2. *Extraction of phospholipid from fibre*

(10 g. fibre (1·98 g. dry matter, 6·13 mg. P) extracted successively as shown in the table.)

	Dry matter		P		
Extractant	(mg.)	(% total in fibre)	(mg.)	(% total in fibre)	(mg./g. dry matter of fraction)
1. Ethanol-ether, 144 ml.	279	14·1	0·216	3·5	0·8
2. 2% (w/v) TCA, 84 ml.	—	—	0·337	5·4	—
3. Ethanol-ether, 98 ml.	85	4·3	1·120	18·1	13·2

Table 3. *Composition of ethanol-ether extracts from fresh and incubated fibre of large and small leaves*

(Two lots (10 g.) of fibre (3·20 g. dry matter, 7·5 mg. P) from large leaves (10–25 cm. long) and 2 lots (10 g.) of fibre (2·35 g. dry matter, 10·9 mg. P) from small leaves (<10 cm. long). The fresh fibres were extracted with 70 ml. 0·2 N-$HClO_4$ and then with 120 ml. ethanol-ether. The incubated fibres were first incubated at 37° overnight in water, then extracted with 0·2 N-$HClO_4$ and ethanol-ether in the same way as the fresh fibres.)

	Large leaves		Small leaves	
	Fresh	Incubated	Fresh	Incubated
Phosphorus:				
mg./g. dry matter of fibre	0·41	0·11	0·70	0·15
% total in fibre	17·4	4·8	15·1	3·3
Nitrogen:				
mg./g. dry matter of fibre	1·8	1·3	2·4	1·4
% total in fibre	3·9	2·8	4·2	2·5
Dry matter (% total in fibre)	15·3	12·4	19·8	14·4

(2) *Ethanol-ether soluble* P. Most of the pigments and about 80% of the lipid material were removed from fibre by extraction with neutral ethanol-ether at room temperature, or with hot solvents in a Soxhlet extractor, without removing more than a small fraction of the phospholipins. After extraction of the fibre with acid, i.e. below pH 2, 15–35% of the fibre P became soluble in cold ethanol-ether. Subsequent extraction with hot solvents showed that extraction in the cold was effective in removing lipid P. A phospholipin fraction with a P content of about 1·5% was obtained by first extracting the fibre with neutral ethanol-ether, then with dilute acid and again with ethanol-ether. Up to 20% of the fibre dry matter was soluble in ethanol-ether. Results are given in Table 2 showing the separation of phospholipin from other lipids.

Incubation of fibre in water decreased the amount of P soluble in ethanol-ether following acid extraction by as much as 90% (Table 1). The dry matter extracted was diminished by less than 20%. Table 3 compares the composition of the ethanol-ether fraction, following acid extraction, from fresh and incubated fibre of large and small leaves.

several hours in acid of the same strength all the P which was brought into solution was in organic form. Lipid P, if not already removed, was extracted in addition to nucleic acid and over 90% of the fibre P was brought out. Ogur & Rosen (1950) found that ribonucleic acid (RNA) could be extracted from root tips, without removing deoxyribonucleic acid (DNA), by soaking in N-$HClO_4$ at 4° for 18 hr. The DNA was then extracted by heating the residue with two lots of 0·5 N-$HClO_4$ for 20 min. at 70°. They used $HClO_4$ instead of TCA which had been used for much of the earlier work on P fractionation in animal tissues, because unlike TCA it has almost negligible absorption in the ultraviolet. With leaf tissues the RNA is not extracted so easily, and either a higher temperature or a higher concentration of acid has to be used. To remove all the RNA it is necessary to use conditions which will cause some of the DNA to become soluble. Fig. 1 shows the amount of P extracted from acid and ethanol-ether treated fibre with three concentrations of $HClO_4$ (3, 1·0 and 0·33 N) at 4 and 16° and also 1·0 N at 37°. DNA was determined in the extracts by the diphenylamine method. The amount of DNA was negligible except in the

3N-$HClO_4$ extract at 16° after 2 hr. and in all the samples of N-$HClO_4$ at 37°. For the routine determination of RNA the fibre was extracted with N-$HClO_4$ at room temperature overnight. However, the amount of RNA extracted under these conditions does not represent the true total. A second extraction with N-$HClO_4$ at 16° will remove a small amount more RNA but some DNA then becomes soluble. In the chloroplast fraction (p. 438), where results are not complicated by the presence of DNA, it is clear that some of the RNA is not brought out with N-$HClO_4$ at 16°, but that a higher temperature had to be used to extract it. Much of the RNA of fibre is due to the presence of chloroplasts so the same is to be expected of the fibre RNA.

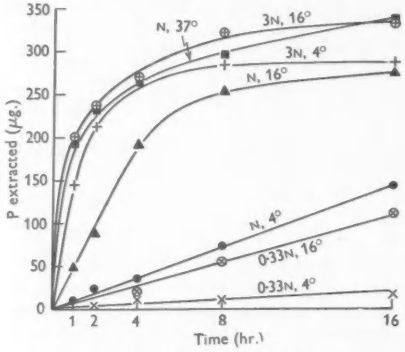

Fig. 1. Extraction of P from fibre with $HClO_4$. 0·5 g. lots of acid and ethanol-ether extracted fibre (dry matter, 141 mg.; P, 410 μg.) in 5 ml. $HClO_4$ (0·33N, N, or 3N) for the times and at temperatures shown.

Less than 5% of the total P in extracts made with N-$HClO_4$ at 16° was inorganic. The carbohydrate content was low, but when 2–3 vol. ethanol were added a precipitate appeared which was mainly 'soluble starch' and pectin. The precipitate contained up to 10% of the P of the extract. When saturated $Ba(OH)_2$ was added to the ethanolic solution to raise the pH to about 9 a precipitate formed containing 60–80% of the P originally present in the extract. The ultraviolet absorption of a N-$HClO_4$ extract had a maximum at about 260 mμ. and absorbed more strongly than an aqueous solution of yeast ribonucleic acid of the same P content. Ogur & Rosen (1950) found that treatment of nucleic acid samples with N-$HClO_4$ increased the absorption at 260 mμ. and this was confirmed using yeast ribonucleic acid.

For extraction of DNA the fibre residue was incubated at 37° with N-$HClO_4$ overnight. One extraction removed the greater part of the P remaining in the fibre, while a second extraction lowered the P content of the residue to a level where determinations became uncertain. A longer extraction at a lower temperature was used instead of the conditions of Ogur & Rosen. This was to minimize degradation of pectic substances because of the interference of galacturonic acid in colorimetric methods of determining DNA (Holden, unpublished). Determinations of DNA by the diphenylamine and tryptophan-$HClO_4$ methods usually agreed fairly well. The results obtained by the cysteine-sulphuric acid method were always lower than by the other two methods. The observed P content of the extract was usually higher than the P to be expected from the colour intensity given by the extract compared with thymus nucleic acid standards of known P content. This is not unexpected for some RNA is present. When agreement between the observed P and the value calculated from deoxyribose determinations was very close it was probably fortuitous, due to interfering substances increasing the absorption at the wavelengths used for colorimetric determinations of deoxyribose.

Extracts made with N-$HClO_4$ at 37° contained more carbohydrate than those made at room temperature, but this was mainly non-reducing. Much of the carbohydrate was precipitated in a bulky gelatinous form, with up to 20% of the P of the extract in it, when 2–3 vol. ethanol were added. From 50 to 70% of the P originally present in the extract was precipitated on addition of saturated $Ba(OH)_2$ to the ethanolic solution.

Nitrogen determinations on N-$HClO_4$ extracts were done after precipitation of $KClO_4$ at 4°, as the presence of large amounts of $HClO_4$ is known to cause loss of N during incineration (Weeks & Friminger, 1942). During the fractionation procedure about 25% of the total N of the fibre was removed, of which about 5% was in the ethanol-ether fraction. There was a greater loss of N when fibre was incubated before fractionation, as up to 15% of the fibre N appeared in the incubation extract. With the usual ratio of fibre to acid each N-$HClO_4$ extract contained about 7% of the fibre N, or 3 mg./g. of the fibre dry matter, whether the fibre had been previously incubated or not.

Incubating fibre in water at 37° diminished the amount of P in the ribonucleic acid fraction by as much as 90%. This is due to the action of ribonuclease present in the fibre (p. 441). There was less diminution in the amount of P in the deoxyribonucleic acid fraction, a loss of from 15 to 35% was found with different batches of fibre. Incubating the fibre in the presence of Mg salts did not cause a greater decrease in the amount of P in this fraction.

Phosphorus fractions in the fibre of leaves of different ages

Pirie (1950) found that the greatest yield of nucleoprotein was from the sap of small young leaves. Table 4 shows the results obtained in one experiment on the fractionation of P in the fibre of leaves of different ages. The leaves were from tall plants, about to flower, and the leaves were grouped according to size and position on the plants. The leaves in the group 'young small' were under 10 cm. long, those in the group 'young large' were 10–20 cm. long and those in the group 'old large' were over 20 cm. long. Some of the leaves in the latter group were slightly yellowed but no completely yellow or withered leaves were included. The total P/g. of the dry matter was highest in the youngest leaves. The acid-soluble P as a percentage of the total P was slightly higher in large leaves than in small but per g. of the dry matter was lower. The inorganic P in this fraction remained fairly constant per g. of the dry matter, but the P in organic form was lower in the large leaves. The phospholipin P/g. of the dry matter was about the same in the different aged leaves, but was a much higher percentage of the total P in the large leaves. Both RNAP and DNAP formed a lower percentage of the total P in the large leaves than in the small and the amount per g. of dry matter was much lower. In small young leaves the RNA content was of the order of 35 mg./g. of the dry matter.

In another experiment the total amount of P in the various fractions in two sizes of leaves was compared. The twelve leaves in each group were chosen to be as uniform in size as possible. In one group, 'small', the leaves were 14 ± 0.5 cm. long, 6.5 ± 0.5 cm. broad at the widest point and weighed 17·3 g. In the other group, 'large', the leaves were 27 ± 1 cm. long, 13.5 ± 1 cm. broad and weighed 74·8 g. The leaves were minced without the midribs being removed and approx. 58 sq.cm. and of the large leaf 240 sq.cm. There were 4·3 times as many cells per unit area in the small leaf as in the large, so that the number of cells was approximately the same. The results for dry matter, N and P values are given in Table 5. The amount of P in the large leaves was greater by only 60%, whereas there was about three times as much dry matter and N. In the fibre the amount of phospholipin P had doubled but the DNAP and RNAP remained the same.

Table 4. *Fractionation of phosphorus in fibre of leaves of different ages*

(3 g. lots of each fibre (known dry matter contents) extracted successively as shown in the table.)

Extractant	P in old, large leaves (mg./g. of dry matter)	P in old, large leaves (% of total)	P in young, large leaves (mg./g. of dry matter)	P in young, large leaves (% of total)	P in young, small leaves (mg./g. of dry matter)	P in young, small leaves (% of total)
1. 0·2 N-$HClO_4$, 30 ml.: inorg. P	0·269	13·1	0·219	11·4	0·247	10·4
org. P	0·115		0·266		0·453	
2. Ethanol-ether, 40 ml. i.e. lipid P	1·02	34·9	0·965	22·7	1·24	18·9
3. N-$HClO_4$ at 16° overnight, 30 ml. i.e. RNAP	1·045	35·6	1·99	46·6	3·16	48·3
4. Two extractions with N-$HClO_4$ at 37° overnight, 40 ml. i.e. mainly DNAP	0·475	16·4	0·811	19·1	1·46	22·4
Totals	2·904	—	4·251	—	6·560	—

Table 5. *Fractionation of phosphorus in leaves of two sizes*

	Twelve large leaves, 74·8 g. wet wt. P (mg.)	N (mg.)	Dry matter (g.)	Twelve small leaves, 17·3 g. wet wt. P (mg.)	N (mg.)	Dry matter (g.)
Sap + fibre washings	6·75	69·5	2·615	3·88	20·0	0·720
Chloroplast fraction	1·87	39·2	0·853	1·06	8·4	0·236
Fibre: Soluble in 0·2 N-$HClO_4$	0·76			0·32		
Soluble in ethanol-ether	1·89			0·88		
Soluble in N-$HClO_4$ at 16°	1·82	101·0	3·34	1·79	42·7	1·175
Soluble in N-$HClO_4$ at 37°	1·13			1·10		
Unextracted	0·22			0·12		
Totals	14·44 = 2·12 mg./g. of dry matter	209·7 = 30·8 mg./g. of dry matter	6·808	9·15 = 4·3 mg./g. of dry matter	71·1 = 33·4 mg./g. of dry matter	2·131

Table 6. *Fractionation of phosphorus in fresh and incubated chloroplast fraction*

(165 mg. lots of a suspension of the chloroplast fraction (3·20 mg. P/g. dry matter) extracted successively as shown in the table. The water extract from the incubated portion was obtained by incubating in water at 37° overnight.)

Extractant	Fresh P (mg./g. dry matter)	Fresh P (% total)	Incubated P (mg./g. dry matter)	Incubated P (% total)
1. Water	—	—	2·25	70·3
2. 0·2 N-$HClO_4$, 11 ml.	0·38	11·9	0·18	5·6
3. Ethanol-ether, 17 ml.	0·93	29·1	0·25	7·8
4. N-$HClO_4$ for 18 hr. at 16°, 13 ml.	1·21	37·9	0·29	9·0
5. N-$HClO_4$ for 18 hr. at 37°, 10 ml.	0·51	16·0	0·12	3·8
Totals	3·03	—	3·09	—

the sap, chloroplast and fibre fractions separated as usual, the washings of the fibre being included with the sap. A count of the number of cells per unit area was made on a representative leaf of each group by the method described by Morton & Watson (1948). The area of the small leaf was

Chloroplast fraction

Table 6 gives the results of a typical experiment on fractionation of P in the chloroplast fraction following the same general scheme as for fibre. The P content per g. of the

dry matter was very variable, depending partly on the age of the leaf and on how much starch was present. The amount of acid-soluble P was about 10% of the total present which is of the same order as that in fibre. The ethanol-ether soluble P was 25–45% of the total in the fraction, which is a higher range than in fibre. As with fibre, most of the phospholipid P was not extractable until after acid treatment.

Extraction with N-$HClO_4$ at room temperature removed about 70% of the P still present after extraction of lipid P; a higher temperature was needed to extract the remainder. One incubation with N-$HClO_4$ at 37° overnight removed all but traces. The acid extracts at room temperature and 37° had ultraviolet absorption spectra similar to that of a yeast ribonucleic acid solution of the same P content in N-$HClO_4$. DNA could not be detected unequivocally by any of the three methods used. The carbohydrate content of the acid extracts was lower than in the corresponding ones from fibre as they contained only starch and no pectin. Nucleotide preparations made by precipitation of the Ba-ethanol insoluble P also contained much less polysaccharide than similar preparations from fibre. The RNA content of the chloroplast fraction from which much of the starch had been removed by differential centrifugation was 30–40 mg./g. of the dry matter.

In the chloroplast fraction which had been incubated in water at 37° the amount of P in the various fractions was greatly diminished owing to the action of ribonuclease and the phospholipid-splitting enzyme. The ribonucleic acid which is not extracted by N-$HClO_4$ at room temperature and which appears to be more firmly bound than the rest, is, however, susceptible to the action of ribonuclease.

Table 7. *Phosphorus fractionation in some sap precipitates*

(200 ml. sap centrifuged at 8000 rev./min. (6000 *g*) to sediment the chloroplast fraction and the successive precipitates formed after standing for 1 and 3 days at 4°. The chloroplast fraction, with much of the starch removed, and the precipitates were washed and then suspended in water. The P fractions were obtained by extracting successively with 0·2 N-$HClO_4$, ethanol-ether and N-$HClO_4$ at 37°.)

	Chloroplast fraction	Ppt. after 1 day	Ppt. after 3 days
Dry matter (g.)	1·62	0·52	0·48
P (mg./g. dry matter)	2·1	2·7	2·8
N (mg./g. dry matter)	46·7	94·3	135
P % of total P in fraction:			
Soluble in 0·2 N-$HClO_4$	16·1	10·4	6·5
Lipid P	47·0	23·8	3·0
Nucleic acid P	36·9	65·8	90·5

Sap fractions

The P was also fractionated by the $HClO_4$ procedure in the following materials prepared from sap: (1) the precipitate obtained by adding $HClO_4$, to a concn. of 0·2 N, to sap after removal of the chloroplast fraction; (2) the precipitates which separated out from sap (after removal of the chloroplast fraction) on standing at 4°; (3) material which sedimented at 8000 rev./min. from the resuspended sediment from sap which had previously been spun at 40000 rev./min. No deoxypentose was detected in any of these fractions. The $HClO_4$ precipitate of sap had a low lipid-P content, 5–10% of the total P. One extraction with N-$HClO_4$ at room temperature removed the greater part of the remaining P, and it was not necessary to incubate at 37° to free the residue of P, a second extraction at room temperature was effective in doing this. Table 7 compares the results of the P fractionation in the chloroplast fraction and the precipitates which appeared in the sap after 1 and 3 days' standing. The lipid P as a percentage of the total P of the fraction decreased in the successive precipitates while the nucleic acid P increased. The material not resuspending after ultracentrifugation had a high P content (over 10 mg./g. of the dry matter), about half of which was nucleic acid P and 40% lipid P. No attempt has been made to fractionate the P in the sap which is not precipitable with acid.

Comparison of methods for estimation of nucleic acids

The method of fractionating RNAP and DNAP used by Schmidt & Thannhauser (1945) on animal tissues and by von Euler & Hahn (1947, 1948) on leaf tissue was tried on several batches of tobacco-leaf fibre and the results compared with the modified Ogur & Rosen method. Incubation of fibre with N-NaOH at 37°, after removal of acid and ethanol-ether soluble P, brought out all the P of the fibre in organic form. However, when fibre was incubated with alkali, even after thorough treatment with solvents to remove pigments, the extract was yellow or brown and unsuitable for colorimetric estimations of inorganic P or of DNA. An added disadvantage was the high N and carbohydrate content of the extracts. The amount of RNAP as a percentage of the total P was higher in the Schmidt & Thannhauser method than in the $HClO_4$ method, while the DNAP was correspondingly lower. This is to be expected because of the DNAP fraction in the $HClO_4$ method containing some RNAP.

FACTORS AFFECTING THE ENZYMIC BREAKDOWN OF PHOSPHORUS COMPOUNDS IN FIBRE, CHLOROPLAST AND SOME SAP FRACTIONS

The release of phosphorus on incubation in water and salt solutions has been observed with fractions from the leaves of a number of species, including tomato (*Lycopersicum esculentum* Mill.), potato (*Solanum tuberosum* L.), bean (*Phaseolus vulgaris* L.), bryony (*Bryonia dioica* Jacq.), comfrey (*Symphytum officinale* L.), groundnut (*Arachis hypogaea* L.), and cabbage (*Brassica oleracea* L.). Most experiments were done with tobacco leaves.

Conditions of incubation

Effect of temperature. At 4° there was a gradual increase in the amount of phosphorus extractable with water. At room temperature the rate of release was increased, and at 37° the action was rapid and substantially complete in a few hours. The greater part of the phosphorus in the extracts was inorganic phosphorus. Fig. 2 shows the effect of temperature on the liberation of P from fibre.

Effect of pH. The optimum pH for the liberation of phosphorus is between 5 and 6. Fig. 3 shows the results of an experiment in which portions of a chloroplast suspension were incubated for 4 hr. at

pH values between 4 and 8. At pH values on the acid side of the optimum the inorganic and total phosphorus in the extracts was of the same order and the amount liberated fell off steeply as the pH became lower. On the alkaline side of the optimum there was a less steep fall in the amount released and the inorganic phosphorus was only half of the total phosphorus in the extracts.

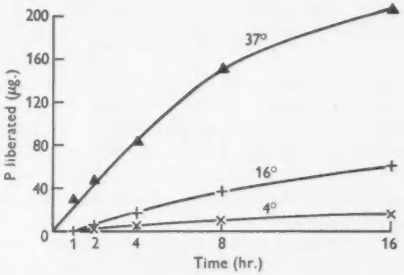

Fig. 2. Effect of temperature on enzymic release of P from fibre. 0·5 g. lots of fibre (dry matter, 100 mg.; P 300 μg.) in 8 ml. 0·025 N-NaCl incubated at 4, 16 and 37° for times shown.

Effect of salts. Sodium chloride, sodium citrate and sodium azide increase the rate of liberation of phosphorus. Table 8 shows the effect of various concentrations of sodium chloride and sodium citrate on the release of phosphorus from fibre. Total and inorganic phosphorus liberation were activated by both salts, but a citrate concentration of only about one-twenty-fifth that of the sodium chloride was needed to achieve the same result. Fig. 4 shows the results for three concentrations of sodium azide, compared with sodium chloride, on liberation of total phosphorus from the chloroplast fraction.

The effects due to the activity of ribonuclease and the phospholipin-splitting enzyme can be separated from each other as the phosphorus is split from the RNA much more rapidly than from the phospholipin, particularly in the presence of citrate. The results of an experiment using fibre are given in Table 9. When the phosphorus fractionation in fresh fibre and in fibre incubated for 1 hr. at 37° in

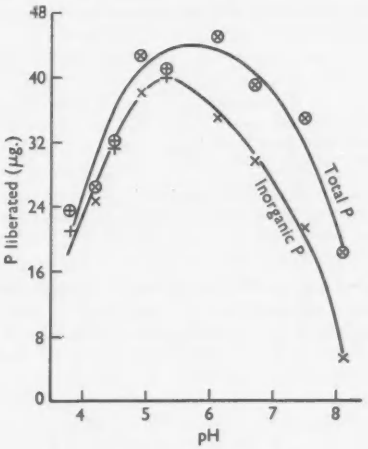

Fig. 3. Effect of pH on enzymic liberation of P from the chloroplast fraction. 1 ml. lots of a suspension of the chloroplast fraction (dry matter, 49 mg.; P, 100 μg.) + 1 ml. buffer solution. The final concentration of the acetate buffers was 0·05 M, and the Michaelis sodium diethylbarbiturate-acetate buffers were a 1 in 10 dilution of the stock solution. Incubated for 4 hr. at 37° and 2 ml. 10% (w/v) TCA added before being centrifuged. Inorganic and total P determined in the supernatants. The P values given by a portion of suspension which was not incubated and to which TCA was added were subtracted from the results. ⊗ and ×, Michaelis buffers; ⊕ and +, acetate buffers.

0·05 M-sodium citrate buffer (pH 6) is compared (*A* and *B* in the table) it is clear that most of the phosphorus in the incubation extract is from the fraction soluble in N-perchloric acid at room

Table 8. *The effect of various concentrations of sodium chloride and sodium citrate on the liberation of phosphorus from fibre*

(0·5 g. lots of fibre (dry matter, 146 mg.; P, 220 μg.) incubated with 5 ml. water or NaCl solution or sodium citrate solution (pH 6) of concn. shown for 2, 4 and 18 hr. at 25°.)

	P in extract (μg.)					
Time of incubation	2 hr.		4 hr.		18 hr.	
	Inorganic	Total	Inorganic	Total	Inorganic	Total
Water	15	22	30	30	54	55
NaCl: 0·25 M	42	58	65	63	109	117
0·05 M	25	27	45	49	76	88
0·01 M	20	22	28	38	60	75
Sodium citrate: 0·05 M	80	120	108	127	151	160
0·01 M	39	53	65	72	135	141
0·002 M	24	24	40	49	67	69

Table 9. *Separation of the effects due to ribonuclease and the phospholipin-splitting enzyme during incubation of fibre*

(3 g. lots of fibre (dry matter 30·2 %, P 2·5 mg./g. of dry matter) (*A*) P fractionated without previous incubation. (*B*) P fractionated after 1 hr. incubation in 0·05 M-sodium citrate buffer solution (pH 6). (*C*) Incubated for 1 hr. as (*B*), extract removed and fibre reincubated for 18 hr. in fresh solution. P fractionated in fibre residue.)

	(*A*)		(*B*)		(*C*)	
	P (mg./g. of dry matter)	P (% total)	P (mg./g. of dry matter)	P (% total)	P (mg./g. of dry matter)	P (% total)
Incubation for 1 hr. at 37°	—	—	1·240	49·5	1·195	48·0
Incubation for 18 hr. at 37°	—	—	—	—	0·508	20·4
0·2 N-$HClO_4$, 25 ml.	0·262	10·5	0·083	3·3	0·083	3·3
Ethanol-ether, 35 ml., i.e. lipid P	0·655	26·2	0·591	23·7	0·161	6·5
N-$HClO_4$, 16°, 25 ml., i.e. RNAP	0·985	39·4	0·117	4·7	0·055	2·2
N-$HClO_4$, 37°, 25 ml., i.e. mainly DNAP	0·497	19·9	0·423	16·9	0·387	15·5

temperature, i.e. RNAP. The lipid phosphorus and DNAP fractions have not been much diminished. When *B* and *C* in the table are compared it is seen

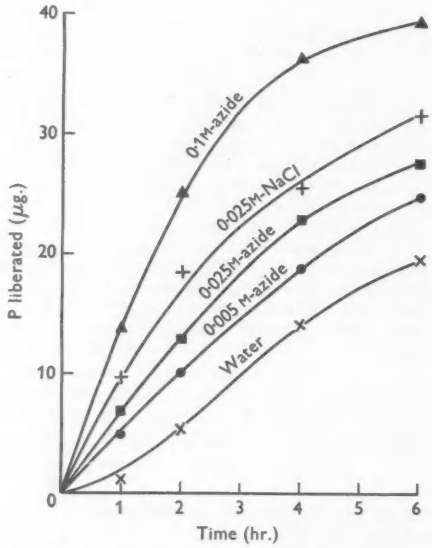

Fig. 4. Effect of sodium azide on enzymic liberation of P from the chloroplast fraction. 1 ml. lots of a suspension of the chloroplast fraction (dry matter, 47 mg.; P, 90 μg.) + 1 ml. water, NaCl or sodium azide solution (with pH previously adjusted to 6·0). Incubated at 37° for times shown and 2 ml. 10 % (w/v) TCA added before being centrifuged. Total P determined in the supernatants. The P value given by a portion of suspension which was not incubated and to which TCA was added was subtracted from the results.

that a second incubation for 18 hr., of another portion of fibre, leads to a further diminution in the RNAP fraction, but that the greater part of the phosphorus in the incubation extract is now from the lipid fraction.

Pretreatments

Milling. Grinding the fibre in a triple roller mill (Bawden & Pirie, 1944) did not increase the rate of liberation of phosphorus or the total amount of phosphorus released when the fibre was subsequently incubated in salt solution at 37°.

Boiling. Fibre which had been boiled did not liberate inorganic phosphorus when it was incubated at pH 6, but some phosphorus in organic form was released. Lipid phosphorus did not decrease in amount when boiled fibre was incubated. The addition of dialysed sap or a dialysed extract from fresh fibre caused inorganic phosphorus to be liberated and the total amount of phosphorus brought out was increased. Table 10 shows the effect of adding a dialysed extract to boiled fibre.

Table 10. *Effect of adding a dialysed fibre extract to boiled fibre*

(0·5 g. lots of fibre (dry matter, 29·3 %; P, 2 mg./g. dry matter) + 4 ml. sodium citrate buffer, 0·02 M (pH 6) + 1 ml. water or 1 ml. dialysed extract of fresh fibre. Incubated at 37° for times given. Total and inorganic P determined in the extracts.)

	P in extract (mg./g. dry fibre)			
	Boiled fibre		Boiled fibre + dialysed extract of fresh fibre	
Time (hr.)	Inorganic	Total	Inorganic	Total
1	<0·02	0·146	0·068	0·247
3	—	0·218	0·102	0·314
6	—	0·287	0·222	0·564
22	<0·02	0·486	0·324	0·966

Acid extraction and ethanol-ether extraction. Phosphorus in organic form, but no inorganic phosphorus, was liberated from fibre which had been extracted with dilute acid or with ethanol-ether when it was subsequently incubated at pH 6.

Enzyme activities of fibre extracts

Extracts made by soaking fibre in sodium chloride or sodium citrate buffer (pH 6), followed by dialysis, had ribonuclease and phosphatase activity. Inorganic phosphorus was also split from nucleotide preparations made from fibre and chloroplast fractions. Citrate did not activate the liberation of organic phosphorus from yeast ribonucleic acid by leaf ribonuclease (Pirie, 1951), nor the enzymic liberation of inorganic phosphorus from sodium β-glycerophosphate and nucleotide preparations. Phosphatase and nucleotidase activity was lost by bringing the enzyme solution to the boil, but the ribonuclease is more heat stable and only about half the activity was destroyed by this treatment. Deoxyribonuclease activity was detectable, but with 24 hr. incubation at 37° only 5 % of the phosphorus from thymus deoxyribonucleic acid was released compared with 75 % from yeast ribonucleic acid. The action of a fibre extract on either lecithin or a phospholipid preparation from leaves has not been tested. The properties of the phospholipid-splitting enzyme are being investigated further.

Composition of incubation extracts

Table 11 gives the composition of a typical incubation extract of fibre. Part of the nitrogen and carbohydrate is present as nucleosides and the remainder as breakdown products of starch and protein due to the activity of the leaf enzymes. No deoxypentose could be determined with certainty in the extracts. The extracts absorbed strongly in the ultraviolet with a maximum at 260 mμ. Adenine, guanine, cytosine and uracil were detected by paper-partition chromatography in the extracts after hydrolysis.

Table 11. *Composition of an incubation extract of fibre*

(6 g. fibre (1·80 g. dry matter, 58 mg. N, 5·4 mg. P) incubated at 37° in 30 ml. water for 18 hr.)

	Total in extract (mg.)
Dry matter	234
Total P	3·33
Inorganic P	2·64
Nitrogen	9·9
Total carbohydrate	102
Reducing sugar	72

DISCUSSION

The observation that most of the phospholipid of leaf fibre and chloroplast fractions is not extracted with ethanol-ether until after treatment with acid is in agreement with results obtained with animal and some other plant tissues (Lovern, 1942). In animal tissues there is evidence that lipids are bound because of the formation of lipoprotein complexes but in plants combination with carbohydrates has also been suggested (Jamieson, 1938). Frey-Wyssling (1949) was of the opinion that lipoproteins were present in intact chloroplasts, but on isolation and preparation the lipids dissociated from the protein. It is clear, however, that it is a stable linkage between the phospholipid and protein or other substance to which it is attached.

Hanahan & Chaikoff (1948) found that an enzyme preparation from cabbage leaves acting on soy bean lecithin caused a loss of only 5 % of the ether-soluble phosphorus, when 35 % of the nitrogen was removed. They suggested that the activity of this enzyme might explain why Chibnall & Channon (1927) isolated a phosphatidic acid from fresh cabbage leaves. In tobacco leaves, and in others including cabbage, phospholipin-splitting enzymes capable of attacking other linkages are also present. The loss of ethanol-ether soluble P from fibre and chloroplast fractions was much greater than the loss of nitrogen on incubation. The different results obtained by Hanahan & Chaikoff might be due to the different substrate used or to a difference in the stability of the enzymes concerned with phospholipin breakdown.

Sodium chloride, sodium citrate and sodium azide have an activating effect on the liberation of phosphorus from fibre and chloroplast fractions similar to that found with the fission of nucleoprotein (Pirie, 1950). Sodium azide appears to act like any other salt. As ribonuclease activity is not much affected by citrate, the effect of citrate on phosphorus liberation may be due to increased accessibility of the nucleic acid. The apparent activation by citrate of nucleotidase action in fibre, which is not observed with nucleotide preparations, is probably due to more substrate being available.

When fibre which had been boiled, or extracted with dilute acid or with ethanol-ether, was subsequently incubated at pH 6 some phosphorus in organic form but no inorganic phosphorus was brought into solution. The release of organic phosphorus was probably due to ribonuclease activity still present in the fibre. The ribonuclease is relatively heat stable but the phosphatase is readily inactivated. Parker (1951) found that TCA precipitates of sap had about 4 % of the ribonuclease activity of the original sap, whereas the phosphatase was almost completely destroyed.

The deoxyribonuclease activity of dialysed sap and fibre extracts was so low that it is not unexpected that the DNAP was affected much less than the RNAP during incubation of fibre. The decrease in the DNAP fraction is probably due mainly to the RNAP present in the fraction being removed by ribonuclease action.

During expansion of the leaf there is a large increase in the total amount of phospholipin present. The dry matter has increased in amount so that phospholipid expressed on a dry matter basis remains the same or increases. The total amount of phosphorus in the nucleic acid fractions from fibre remains about the same, so that it shows a decrease per g. of the dry matter. If the weight of DNA per cell is constant in amount as has been found for various animal tissues (Vendrely & Vendrely, 1948) and bacteria (Mitchell & Moyle, 1951), it would not be expected that DNA would vary during expansion of the leaf as cell division is not taking place.

The perchloric acid method of fractionating phosphorus compounds, although not giving a perfect separation of ribo- and deoxyribo-nucleic acids in leaf tissue, is useful in giving an RNA fraction substantially free from DNA, though the DNA fraction contains RNA. The fact that some of the RNA is not extracted by perchloric acid at room temperature, but requires a higher temperature to bring it out, suggests that it might be bound in a different way from the more easily extracted fraction.

Of the total P in the leaf about 30 % is in the form of ribonucleic acid phosphorus, two-thirds of which is in the fibre. Deoxyribonucleic acid phosphorus accounts for about 7 % and lipid phosphorus a further 15 % of the total phosphorus of the leaf.

SUMMARY

1. The phosphorus of tobacco-leaf fibre and chloroplast fractions has been fractionated into acid-soluble phosphorus, lipid phosphorus, ribonucleic acid phosphorus and deoxyribonucleic acid phosphorus. The distribution in leaves of different ages has been compared.

2. Incubation of fibre and chloroplast fractions in water and salt solutions leads to the liberation of up to 80 % of the total phosphorus present, owing to the activity of ribonuclease and a phospholipin-splitting enzyme.

3. The optimum pH for the enzymic liberation of phosphorus is between 5·5 and 6.

4. Sodium chloride, sodium azide and sodium citrate activate the enzymic release of phosphorus.

5. Milling the fibre does not increase the rate of liberation or the total amount of phosphorus released by enzymic action.

6. Boiled fibre, acid-extracted fibre and ethanol-ether extracted fibre on incubation at pH 6 release phosphorus in organic form but no inorganic phosphorus. When incubated in the presence of dialysed sap or a fibre extract, which have ribonuclease and phosphatase activity, inorganic phosphorus is released and the total phosphorus liberated is increased.

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Reactions of Haematins with Peroxides

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It is well known that hydrogen peroxide reacts with haemoproteins such as methaemoglobin, catalase and peroxidase, giving a series of compounds which can be distinguished by their absorption spectra. In recent years much work has been done on the mechanism of these reactions, and it was found that catalase and peroxidase are each capable of forming at least three distinct complexes with hydrogen peroxide. The properties of these complexes and the literature on the subject of their formation has recently been reviewed by Theorell (1947), Lemberg & Legge (1949), and Keilin & Hartree (1951). Since in all these complexes it is the haematin prosthetic group which reacts directly with the peroxide, it was of interest to find whether free haematins can combine with peroxide. So far the only attempt to study this problem was made by Haurowitz (1937*a*, *b*), whose observations on the reaction between protohaematin and hydrogen peroxide in pyridine will be discussed later in the light of the findings reported here.

In this paper it is proposed to examine the conditions under which free haematins react with hydrogen peroxide and ethyl hydroperoxide and the nature of the products of these reactions, the experiments being carried out both with urohaemin and protohaemin. Urohaemin was selected for this purpose because, being much more soluble than protohaemin on account of its eight carboxyl side chains, it had been found to react with many substances such as sodium hydroxide and amino-acids with much greater ease than protohaemin (Keilin, J., 1949, 1950).

EXPERIMENTAL

Protohaemin. This was prepared from ox or horse blood by the method of Schalfejew (1885).

Urohaematin. Uroporphyrin I was isolated from the urine of a case of congenital porphyria. The uroporphyrin was esterified, and urohaemin was prepared from the octamethyl ester according to the method of Fischer & Orth (1934). A $6{\cdot}1 \times 10^{-4}$M solution of urohaematin in 0·02N-NaOH was obtained by dissolving the urohaemin in 5 ml. 0·1N-NaOH, and the volume was made up to 25 ml. with distilled water.

Hydrogen peroxide. The molarity of H_2O_2 in a solution containing 1 ml. perhydrol in 250 ml. distilled water was determined by titration with standard $KMnO_4$ in the usual way. Further dilutions of the H_2O_2 solution were made as required daily. A fresh stock solution was prepared and standardized every few days.

Ethyl hydroperoxide. This was prepared and standardized according to the method of Baeyer & Villiger (1901).

Spectroscopic methods. The direct observations of the reactions were carried out with a microspectroscope. The absorption spectra in the visible and violet regions were determined with either the Beckman photoelectric spectrophotometer or the Hilger Uvispek photometer; the latter instrument was used for measuring the high absorption densities in the region of the Soret band. The molecular absorption coefficient ϵ is defined as follows: $\epsilon = E/cl$, where c = molarity of the haematin solution, l = optical depth in cm., and E (extinction) = $\log I_0/I$, where I_0 and I are the intensities of the incident and transmitted light respectively.

RESULTS

The absorption spectrum of the urohaematin-H_2O_2 complex

When 0·5 ml. of a $6{\cdot}1 \times 10^{-4}$M solution of urohaematin in 0·2N-NaOH is treated with an equal volume of 0·27M-H_2O_2 in the presence of 2 ml. N-NaOH, the colour of the solution at once changes from reddish brown to salmon-pink. At the same time, on direct spectroscopic examination, it is seen that the absorption band of urohaematin at 594 mμ. is replaced by two well defined bands in the green region of the spectrum, of which the β-band is a little stronger than the α-band. This absorption spectrum is, however, of very short duration, and within 45–60 sec. the urohaematin band at 594 mμ. begins to reappear as a shading in the yellow region of the spectrum. This band gradually becomes stronger, and the two bands in the green region diminish in intensity as the complex reverts to free urohaematin. These changes, although they begin to take place very soon after the complex is formed, slow down after about 1·5–2 min. and thereafter progress more slowly so that the peroxide complex takes several hours to revert completely to free urohaematin (Fig. 1). At the same time some molecular oxygen is liberated from the H_2O_2, and there is also some peroxidatic destruction of the urohaematin itself.

In view of the rapid onset of changes in the absorption spectrum of the complex, a modified technique had to be adopted for recording the spectrophotometric curve and the urohaematin-peroxide complex was freshly made for each point of the curve. The following procedure was found to

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be the most convenient: 0·5 ml. of a $6·1 \times 10^{-4}$M solution of urohaematin was placed in a 1 cm. cell and diluted with 2 ml. N-NaOH (final concentration of NaOH = 0·7N). After adjusting the instrument at the selected wavelength with the control cell in position, 0·5 ml. of a 0·27M solution of H_2O_2 (450 mol. H_2O_2/mol. urohaematin) was added at zero time by means of an all-glass syringe fitted with a stainless steel needle and the contents of the cell were stirred with a glass rod. Three to four readings of the extinction were taken within the first 2 min.; readings were then taken at 1 min. intervals for the next 5 or 10 min., so long as O_2 bubbles in the solution did not obscure the optical surfaces of the cell or could be dislodged from them by tapping the cell between readings. The changes of extinction were plotted against time and the spectrophotometric curves were constructed from the extinction obtained by extrapolating back to zero time, assuming the reaction to take place instantaneously on the addition of the hydrogen peroxide to the urohaematin. To determine the absorption spectrum of the Soret band, 0·25 ml. H_2O_2 (750 mol. H_2O_2/mol. urohaematin) was added to 0·15 ml. urohaematin diluted with 2·6 ml. of the appropriate alkali. The rest of the operation was carried out as described for the visible region of the spectrum.

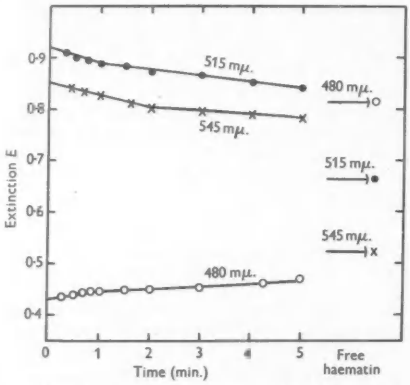


Fig. 1. Reaction of urohaematin with H_2O_2 in 0·7N-NaOH. Typical changes in extinction with time at three wavelengths (515, 545 and 480 mμ.) showing the gradual reversion of the absorption spectrum of the compound to that of free haematin, indicated by the separate points on the right of the figure. The values of $\epsilon \times 10^{-4}$ used for Fig. 2 were obtained by extrapolating back to zero time. (ϵ as defined in text.)

The spectrophotometric curves of the urohaematin-peroxide compound obtained in this way show that the positions of the two bands in the visible region of the spectrum are α—547·5 mμ. and β—521 mμ., while the values of $\epsilon \times 10^{-4}$ are 0·87 and 0·95 respectively. In the violet region of the spectrum the Soret band of the urohaematin at 395 mμ. is replaced by the single symmetrical band of the peroxide compound at 413 mμ. ($\epsilon \times 10^{-4} = 7·9$) (Figs. 2 and 3).

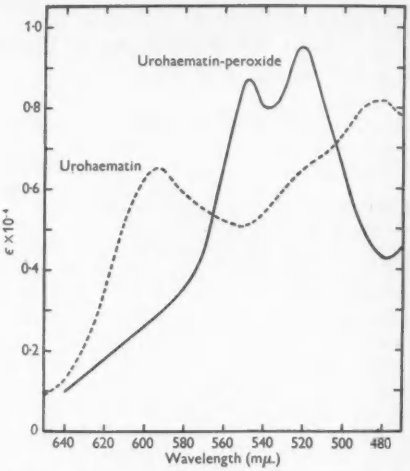


Fig. 2. Absorption bands in the visible region of the spectrum of urohaematin and urohaematin-peroxide in 0·7N-NaOH. (Urohaematin = $1·02 \times 10^{-4}$M; $H_2O_2 = 4·5 \times 10^{-2}$M; $l = 1$ cm.)

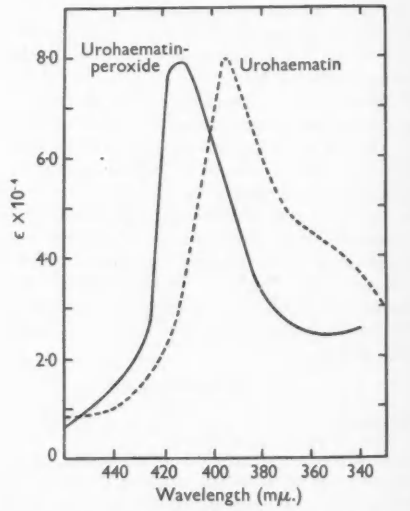


Fig. 3. Absorption spectra showing the Soret bands of urohaematin and urohaematin-peroxide in 0·7N-NaOH. (Urohaematin = $3·05 \times 10^{-5}$M; $H_2O_2 = 2·25 \times 10^{-2}$M; $l =$ 1 cm.)

Stoicheiometric relationships

In order to determine the number of molecules of H_2O_2 combined with each molecule of urohaematin, the urohaematin was treated with various concentrations of H_2O_2 in the presence of 0·7 N-NaOH under standard conditions and the percentage formation of the urohaematin-peroxide compound formed in each case was determined as follows. To 0·5 ml. of a $6{\cdot}1 \times 10^{-4}$ M solution of urohaematin in 0·02 M-NaOH in a 1 cm. optical cell, were added 2 ml. M-NaOH and the calculated amount of distilled water required to bring the total volume of the solution to 3 ml. after the addition of the H_2O_2. A dilute solution of H_2O_2 was introduced into the cell at zero time by means of a syringe so as to provide concentrations of H_2O_2 in the solution ranging from $2{\cdot}05 \times 10^{-4}$ to $4{\cdot}65 \times 10^{-2}$ M, i.e. from 2 to 465 mol. H_2O_2/mol. urohaematin. The extinction at 594 mμ. was then measured at intervals during the first 2 min. and the value at zero time was obtained by extrapolation as described in the previous section. From these values the percentage formation (x) of the peroxide compound at each concentration of H_2O_2 was calculated by means of the formula $x = 100(a-c)/(a-b)$, where a = extinction of free urohaematin, b = extinction of the fully formed urohaematin-peroxide (465 mol. H_2O_2/mol. urohaematin), and c = extinction of the solution under investigation, all the extinctions being measured at the same wavelength (594 mμ.).

If the reaction is represented by the following equation:

$$\text{Urohaematin} + n\text{H}_2\text{O}_2 \rightleftharpoons \text{Urohaematin } (\text{H}_2\text{O}_2)_n,$$

then by the law of mass action

$$K = \frac{[\text{Urohaematin}]\,[\text{H}_2\text{O}_2]^n}{[\text{Urohaematin } (\text{H}_2\text{O}_2)_n]}.$$

Hence if x represents the percentage of the total urohaematin which is combined with H_2O_2, then

$$\frac{1}{K} = \frac{x}{(100-x)\,[\text{H}_2\text{O}_2]^n},$$

whence

$$\log [x/(100-x)] = n \log [\text{H}_2\text{O}_2] - \log K.$$

By plotting $\log [x/(100-x)]$ against $\log [H_2O_2]$ a straight line can be obtained which has a slope of n. When $x = 50$,

$$\log [x/(100-x)] = 0 \quad \text{and} \quad \log K = n \log [\text{H}_2\text{O}_2].$$

Thus the value of $\log H_2O_2$ when

$$\log [x/(100-x)] = 0, \quad \text{equals} \quad 1/n \log K.$$

Since n can be calculated from the slope of the line, $\log K$ can be obtained. The position of the line and the values of n and K were obtained by applying the method of least squares to the experimental results after a correction had been made for H_2O_2 combined with urohaematin (Table 1, Fig. 4). It was found that 1 molecule of peroxide is combined per molecule of urohaematin ($n = 1{\cdot}085$) and the dissociation constant $K = 3{\cdot}34 \times 10^{-4}$ M at 20°.

Table 1. *The relation between the concentration of H_2O_2 and percentage formation of urohaematin-peroxide as determined experimentally*

Molecular ratio H_2O_2 to urohaematin	Formation of complex (%) (x)	Free urohaematin (%) ($100-x$)	$\log\left(\frac{x}{100-x}\right)$	Total H_2O_2 concn. (M)	Free H_2O_2 concn. (M)	Free H_2O_2 concn. (log M)
2	22·8	77·2	−0·5287	$2{\cdot}046 \times 10^{-4}$	$1{\cdot}811 \times 10^{-4}$	−3·7423
5	38·0	62·0	−0·2125	$5{\cdot}115 \times 10^{-4}$	$4{\cdot}707 \times 10^{-4}$	−3·3273
10	55·8	44·2	+0·1011	$1{\cdot}023 \times 10^{-3}$	$9{\cdot}66 \times 10^{-4}$	−3·0150
15	77·1	22·9	+0·5250	$1{\cdot}534 \times 10^{-3}$	$1{\cdot}455 \times 10^{-3}$	−2·8371

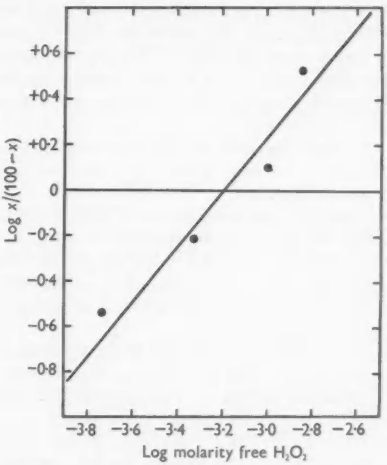


Fig. 4. Relationship between log molarity H_2O_2 and $\log [x/(100-x)]$, where x = percentage formation of urohaematin-peroxide, and $(100-x)$ = percentage of free urohaematin. The experimental results are indicated by dots. The position of the line was obtained by applying the method of least squares to the experimental results. Correction has been made for the H_2O_2 combined with the urohaematin (Table 1).

Conditions required for the formation of the urohaematin-H_2O_2 complex

If urohaematin in 0·2N-NaOH is treated with H_2O_2 in the presence of 0·1N-NaOH instead of N-NaOH as described above, the complex formation never proceeds to completion and a shadow due to the persistence of the free urohaematin band is always seen in the yellow region of the spectrum. A spectrophotometric curve constructed under these conditions (total concentration of NaOH = 0·07N) showed that both bands were nearer the blue end of the spectrum and their relative intensities were reversed (α—544·5 mμ., $\epsilon \times 10^{-4} = 0{\cdot}9$; β—516 mμ., $\epsilon \times 10^{-4} = 0{\cdot}845$). The urohaematin band persisted as a shoulder about 594 mμ. and from the extinction readings at this wavelength it was calculated that only 83% of the urohaematin had reacted with H_2O_2. On the addition of more H_2O_2 to such a solution in an attempt to produce 100% formation of the complex, the colour of the solution turned to greenish brown and the absorption bands disappeared, indicating that the urohaematin was destroyed.

Similar results are observed using the microspectroscope when the total concentration of NaOH is 0·016N or when 0·1 or 0·28M-Na_2CO_3 is used as diluting fluid instead of NaOH. If, however, under these conditions a few drops of N-NaOH are added the complex formation proceeds to completion.

Urohaematin dissolved in 0·1M-phosphate buffer, pH 7·3, fails to react with H_2O_2 and is, instead, rapidly destroyed. The same results are obtained when the solution is made more alkaline by the addition of disodium phosphate or Na_2CO_3 (0·1, 0·28 or 1·4M), but the addition of NaOH to urohaematin in phosphate buffer allows the peroxide compound to be formed as already described.

When urohaematin is dissolved in 10% (v/v) ammonia, the crimson solution of ammonia uroparahaematin shows a very broad and diffuse absorption band in the green region of the spectrum extending from 580 to 510 mμ. with a sharper component centred at 540 mμ. On the addition of H_2O_2 (50 mol. H_2O_2/mol. urohaematin) the colour of the solution changes to a scarlet red and the two bands of the peroxide compound appear, only to give way in a few minutes to the absorption band of the parahaematin. If sodium dithionite ($Na_2S_2O_4$) is added to the urohaematin-peroxide compound in ammonia, the intense absorption bands of the ammonia haemochromogen immediately appear, whereas reduction of urohaematin-peroxide in NaOH gives rise to dihydroxyl-haem (Keilin, J., 1949).

Reactions of pyridine uroparahaematin with H_2O_2

Urohaemin is not readily soluble in pyridine, and the pyridine parahaematin is only obtained in aqueous solution at approximately neutral or slightly acid pH. The red solution then shows the characteristic two-banded spectrum of a parahaematin in which the β-band is stronger and broader than the α-band (α—555 mμ., $\epsilon \times 10^{-4} = 0{\cdot}69$; β—525 mμ., $\epsilon \times 10^{-4} = 0{\cdot}92$). On the addition of H_2O_2 to the parahaematin at about pH 7 the red

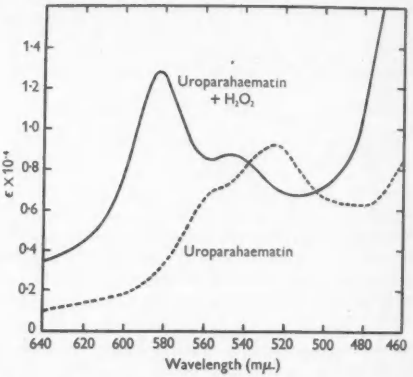


Fig. 5. Absorption bands in the visible region of the spectrum of pyridine uroparahaematin and the green compound given by it on the addition of H_2O_2. (Uroparahaematin = $1{\cdot}045 \times 10^{-4}$M; $H_2O_2 = 4{\cdot}5 \times 10^{-3}$M; l = 1 cm.)

solution quickly turns brown and appears green when viewed in a thin layer. Direct spectroscopic observation shows that this colour change is accompanied by the disappearance of the parahaematin absorption bands. After a slight lag of about 15 sec. during which no absorption bands are visible, two bands appear and gradually grow in intensity. The α-band at 582 mμ. becomes very strong while the β-band at 547 mμ. remains weak (Fig. 5). This absorption spectrum is stable and, so long as excess H_2O_2 is not present to bleach the solution, it may remain for at least 24 hr. At pH 4 these absorption bands appear more intense, while the addition of NaOH causes them to become weaker and finally to disappear. The addition of $Na_2S_2O_4$ to the fully formed green compound at either acid or alkaline pH does not result in the formation of any pyridine haemochromogen, and there is no spectroscopic evidence that the compound combines with CO (either before or after the addition of $Na_2S_2O_4$) or is affected by potassium ferricyanide.

The spectrophotometric curve of the green peroxide-treated parahaematin ('green compound')

was determined as follows: to 0·5 ml. of $1·045 \times 10^{-4}$ M-urohaematin in 0·02 N-NaOH were added 0·75 ml. of 0·02 M-acetic acid, 0·5 ml. pyridine and 0·5 ml. of 0·027 M-H_2O_2 and the volume of the solution (containing 50 mol. H_2O_2/mol. haematin) was brought up to 3 ml. with distilled water; for pyridine uroparahaematin the H_2O_2 was replaced by distilled water. The spectrophotometric curves were determined in the usual way in the visible region, but in the case of the green compounds it was found that, be fully discussed later, it was decided to re-examine the effect of H_2O_2 on protohaematin in pyridine under various conditions.

Protohaemin dissolves readily in pyridine giving a red parahaematin with the usual two-banded absorption spectrum (α—560 mμ., β—530 mμ.). On the addition of H_2O_2 (perhydrol diluted as required with pyridine), the solution becomes greenish brown and a diffuse absorption band appears at about 605 mμ. while the α-band of the

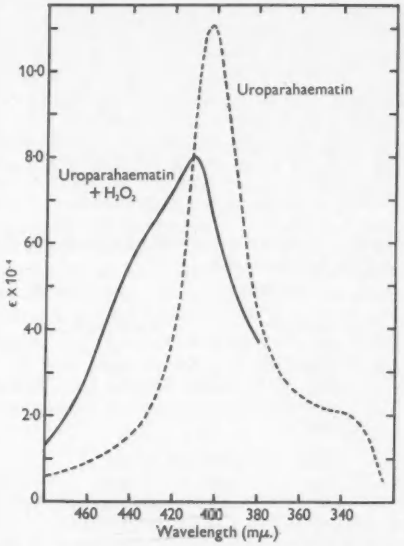


Fig. 6. Absorption spectra showing the Soret bands of pyridine uroparahaematin and the green compound given by it on the addition of H_2O_2. (Uroparahaematin = $1·016 \times 10^{-4}$ M; $H_2O_2 = 2·97 \times 10^{-3}$ M; l = 1 cm.)

at the much greater dilution required for measuring the Soret band, this band was very evanescent. A fresh solution therefore had to be prepared for measuring each point of the curve when determining the height of the Soret band and the peroxide was added last to the cell by means of a syringe as described earlier in this paper. Even with these precautions it was not possible to obtain reliable extinction readings. As seen in Fig. 6, the asymmetric Soret band of the 'green compound' has a main peak at 410 mμ. ($\epsilon \times 10^{-4} = 8·0$ approx.) and a shoulder at 435 mμ. which may be due to some intermediate compound.

Reactions of protohaemin and H_2O_2 in pyridine

In view of the differences between the reactions of urohaematin with H_2O_2 described above and the results obtained by Haurowitz (1937*a*, *b*) which will

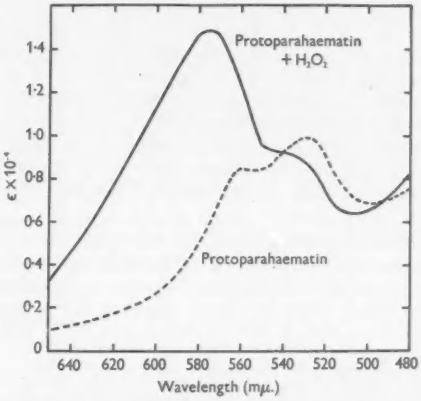


Fig. 7. Absorption bands in the visible region of the spectrum of pyridine protoparahaematin and the green compound obtained on treating it with H_2O_2. (Protoparahaematin = $1·32 \times 10^{-4}$ M; $H_2O_2 = 1·86 \times 10^{-2}$ M; l = 1 cm.)

parahaematin becomes stronger and the β-band at 530 mμ. becomes weaker. The band in the red then tends to become stronger and broader and to move towards the blue end of the spectrum so that its centre lies at about 583 mμ. The general appearance is therefore of a two-banded spectrum with a broad, strong band at 583 mμ. and a weaker band at 560 mμ., but the exact positions of these bands depend on both the pH of the solution and the amount of H_2O_2 added. Thus at pH 6·5 the solution is green with red dichroism; the α-band is markedly asymmetric and appears to extend from 570 to 600 mμ. on direct spectroscopic examination, though the spectrophotometric curve shows that the peak lies at 574 mμ. (Fig. 7). A weak β-band lies at 535 mμ., while in the violet region the Soret band of the parahaematin at 403·5 mμ. is replaced by the band of the 'green compound' at 426 mμ. On reduction with $Na_2S_2O_4$ a haemochromogen (α—558 mμ., β—530 mμ.) is formed by that part of the protohaem which has not been irreversibly altered by the peroxide. With greater concentrations of H_2O_2 the bands at 583 and 560 mμ. appear more

directly and soon disappear due to the destruction of the protohaematin. Under such conditions, therefore, no haemochromogen is obtained on the addition of $Na_2S_2O_4$.

Reactions between ethyl hydroperoxide and haematins

Ethyl hydroperoxide (EtOOH) reacts with haemoproteins giving, in most cases, complexes which are analogous to those obtained with H_2O_2, so it was of interest to examine the effect of EtOOH on uro- and proto-haematin.

With urohaematin in NaOH, EtOOH gave an unstable red compound with two absorption bands in the visible region of the spectrum in the same positions as those of the urohaematin-OOH compound. This spectrum could already be observed in solutions containing 0·02 N-NaOH, but the concentration of NaOH in the solution had to be raised to 1·5 N before the compound was completely formed, as shown by the disappearance of the band of free strong, wide β-band at 546 mμ. The reduction of urohaematin-OOEt to dihydroxyl-urohaem can, of course, be brought about at once by the addition of $Na_2S_2O_4$ to the compound.

With pyridine uroparahaematin, EtOOH reacted somewhat differently from H_2O_2. The scarlet colour of the parahaematin gave way to a greenish brown; at the same time the absorption bands of the parahaematin became diffuse, disappeared and were replaced by two bands, a strong α-band at 575 mμ. and a weak β-band at 537 mμ. After standing a few minutes (or immediately on the addition of $Na_2S_2O_4$) these bands were replaced by a wide asymmetric α-band lying at 560 mμ. and a weak β-band at 527 mμ. belonging to a reduced compound. This absorption spectrum remained unchanged in the presence of CO thus indicating that it was not a true haemochromogen, and it reverted to the oxidized form on the addition of potassium ferricyanide (Table 2).

Table 2. *Positions and extinction coefficients of the α-, β- and γ-bands of the compounds of urohaematin and protohaematin with H_2O_2 and ethyl hydroperoxide*

Compound	α-Band		β-Band		γ-Band	
	Wavelength (mμ.)	$\epsilon \times 10^{-4}$	Wavelength (mμ.)	$\epsilon \times 10^{-4}$	Wavelength (mμ.)	$\epsilon \times 10^{-4}$
Urohaematin in 0·7 N-NaOH	594	0·65	483	0·82	395	8·0
Urohaematin in 0·7 N-NaOH + H_2O_2	547·5	0·87	521	0·95	413	7·9
Urohaematin in 1·5 N-NaOH + EtOOH	547·5*	—	521*	—	—	—
Reduction compound of urohaematin in 1·5 N-NaOH + EtOOH	578*	—	546*	—	—	—
Dihydroxyl urohaem†	578	0·76	546	1·4	435	11
Pyridine uroparahaematin	555	0·69	525.	0·92	402	11·05
Pyridine uroparahaematin + H_2O_2	582	1·27	547	0·87	410	8·0
Pyridine uroparahaematin + EtOOH	575*	—	537*	—	—	—
Reduction compound of pyridine uroparahaematin + EtOOH	560*	—	527*	—	—	—
Pyridine urohaemochromogen (pH 7)	550*	—	520*	—	—	—
Pyridine protoparahaematin	(560	0·84	530	0·99	403·5	6·55
Pyridine protoparahaematin + H_2O_2 (pH 8 approx.)	605)* 583*	—	560	—	—	—
Pyridine protoparahaematin + H_2O_2 (pH 6·5)	574	1·48	535	0·91	426	5·0
Pyridine protoparahaematin + EtOOH (pH 7·8)	(605)* 562*	—	530*	—	—	—
Pyridine protohaemochromogen	558*	—	530*	—	—	—

* Positions of bands determined with microspectroscope. † Keilin, J., 1949.

urohaematin at 594 mμ. Increasing the amount of EtOOH at lower concentrations of NaOH merely led to the destruction of the urohaematin.

When the red urohaematin-OOEt compound was formed under optimal conditions and allowed to stand, the following changes took place within about 3 min. The absorption bands of the compound faded, the solution becoming orange-pink in colour, and they were then gradually replaced by the characteristic absorption spectrum of the red ferrous compound, dihydroxyl-urohaem, which consists of a narrow, weak α-band at 578 mμ. and a

Since ethyl hydroperoxide decomposes spontaneously to give acetaldehyde and water, it was necessary to examine the action of acetaldehyde on various urohaematin compounds before attributing to EtOOH the spontaneous reductions of urohaematin-OOEt and the green compound given by the parahaematin. Acetaldehyde was therefore added to alkaline urohaematin and to pyridine uroparahaematin but no changes were observed in the absorption spectra of these compounds.

In the case of protohaematin no reaction was observed between EtOOH and alkaline protohaematin,

although various concentrations of NaOH and both aqueous and ethanolic solutions of protohaematin were examined. Ethanolic solutions were investigated, since it appeared likely that a haematin requires for its reaction with peroxide the same conditions as are necessary for it to give a dihydroxyl haem in its reduced state (protohaematin can only react with NaOH to give its dihydroxyl compound in the presence of a solvent such as ethanol). This point will be discussed more fully later.

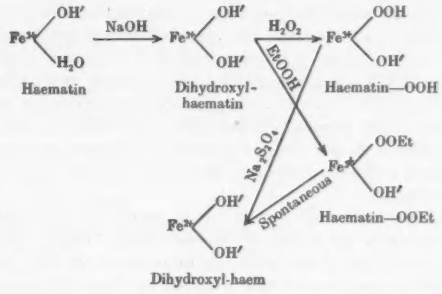
Ethyl hydroperoxide did, however, react with pyridine protoparahaematin. At pH 7·8 the parahaematin solution became greenish brown on the addition of EtOOH and an absorption band was seen for about 30 sec. at 605 mμ. Meanwhile, the band at 560 mμ. became stronger and the parahaematin spectrum was spontaneously replaced by that of a fairly stable green compound with α- and β-bands at 562 and 530 mμ. respectively, the change being complete within a few minutes. This compound, which failed to react with CO and was not affected by $Na_2S_2O_4$, is probably analogous to the reduction compound given by pyridine uroparahaematin and EtOOH. If less EtOOH were used, the reaction was complicated by the final appearance of a haemochromogen (α—558 mμ., β—530 mμ.) given by some of the haematin which had not been altered by EtOOH. This haemochromogen reacted normally with CO.

DISCUSSION

Urohaematin, although very freely soluble in all dilute alkalis and alkaline buffer solutions, was found to react with H_2O_2 and with ethyl hydroperoxide to form reversible compounds only when excess hydroxyl ion was present as in sodium hydroxide solutions. A well defined, though unstable, compound was then formed with two absorption bands in the visible region of the spectrum (α—547·5 mμ., β—521 mμ.). This absorption spectrum was of relatively short duration, and in the case of the H_2O_2 compound it reverted to that of free urohaematin. Titration of urohaematin with H_2O_2 showed that one molecule of H_2O_2 is combined per molecule of urohaematin.

It is now generally accepted that in alkaline haematins the iron atom is co-ordinated in positions 5 and 6 with one hydroxyl group and one molecule of water (Davies, 1940; Shack & Clark, 1947), whereas in haems (ferroporphyrins) these places are occupied by 2 molecules of water. It was recently shown (Keilin, 1949) that under certain conditions in the presence of NaOH, haems react with the hydroxyl ion giving dihydroxyl-haems in which two hydroyxl groups are co-ordinated per atom of haem iron.

Since, for the formation of the peroxide compound, urohaematin also requires the presence of excess hydroxyl ion, and reduction of the urohaematin-peroxide compound with $Na_2S_2O_4$ gives dihydroxyl-urohaem, it is suggested that a dihydroxyl-urohaematin may be an intermediate in the reaction between urohaematin and H_2O_2, one molecule of H_2O_2 replacing a hydroxyl group on the urohaematin iron. As ethyl hydroperoxide gives a compound with alkaline urohaematin analogous to that given by H_2O_2 and under the same conditions, one may assume that the mechanism of formation of the peroxide compound is the same in each case. It was found, however, that urohaematin-OOEt is spontaneously reduced to dihydroxyl-urohaem in a few minutes. Since acetaldehyde, the decomposition product of ethyl hydroperoxide, cannot cause this reduction one must conclude that it is probably due to the reducing action of ethyl hydroperoxide itself. As there is no ferrous form of urohaematin-peroxide a change in the valency of the urohaematin iron must result in the formation of dihydroxyl-urohaem. In the following scheme, which summarizes these reactions, the lines joining the urohaematin iron to the reacting groups do not indicate the nature of the valency bonds and the four valencies directed towards the pyrrol nitrogen atoms are omitted.



So far a protohaematin-peroxide compound analogous to that given by urohaematin has not been obtained, but this does not preclude its existence and it is possible that the conditions for its formation may yet be found.

Haurowitz (1937*a*, *b*), in his important investigations on the reactions between protohaematin and H_2O_2 in pyridine, described a transient two-banded absorption spectrum (α—582 mμ. and β—573 mμ. approx.) which was replaced by a single asymmetrical band in the visible region at 575 mμ. On the basis of these absorption bands Haurowitz postulated the existence of two protohaematin peroxide compounds in which a molecule of H_2O_2 replaced in turn each of the 2 molecules of pyridine of the parahaematin, thus giving a 'primary' and 'secondary' compound respectively.

It was mentioned earlier that in alkaline solution the addition of H_2O_2 to pyridine-protoparahaematin caused an absorption band to appear at about 583 mμ. while the parahaematin α-band at 560–565 mμ. was reinforced and, together with the new band, presented the appearance of a two-banded spectrum. It is probably this absorption spectrum which was observed by Haurowitz but ascribed by him to the 'primary compound'. The band at 575 mμ., which he attributed to the 'secondary compound', corresponds to that given best by proto-parahaematin with H_2O_2 in neutral or slightly acid conditions (Fig. 7); the fact that this band is asymmetrical and is shifted nearer to the blue end of the spectrum than that of the green compound given by uroparahaematin with H_2O_2 may indicate that it is due to a reduction compound similar to that given by ethyl hydroperoxide with both uro- and proto-parahaematins.

The actions of H_2O_2 on the pyridine-parahaematins of uro- and proto-haemins resulting in the formation of the green compounds are completely different from their actions on urohaematin in NaOH. The non-reversibility of formation of the green compounds, together with the fact that when fully formed they fail to give typical pyridine haemochromogens on the addition of $Na_2S_2O_4$, suggests that the haematin has been irreversibly modified in some way, although the presence of a Soret band indicates that the ring has not actually been opened. These green compounds therefore cannot be considered as haematin-peroxide compounds in which the peroxide is co-ordinated with the haematin iron, as was suggested by Haurowitz, but more probably belong to the oxyporphyrin class of pigments.

The reducing action of a peroxide was first described by Kuhn & Wassermann (1933), who showed that ferric salts can be reduced by H_2O_2 to ferrous salts which are detected by their ability to combine with 2:2′-dipyridyl to give the well known red complex. In the course of their work on azide catalase, Keilin & Hartree (1945) showed that H_2O_2 can reduce azide catalase to a well defined ferrous compound, and recently they have shown that the methaemoglobin-peroxide complex may, by further treatment with H_2O_2, be reduced to haemoglobin which combines with O_2 liberated during the reaction to form oxyhaemoglobin (Keilin & Hartree, 1951). However, on treating methaemoglobin with very small concentrations of H_2O_2 below pH 5, the absorption bands of methaemoglobin disappeared and were replaced by a wide band at 590 mμ. belonging to a new compound showing red-green dichroism, which could be reduced by $Na_2S_2O_4$ to haemoglobin. From a consideration of its spectroscopic properties, Keilin & Hartree concluded that it was an oxidation product of haemoglobin in which the haematin ring, though still intact, was modified, probably at one of the methine bridges. This compound bears a strong resemblance to that obtained by treating pyridine protohaematin with H_2O_2. Thus H_2O_2 can be both a reducing agent and an oxidizing agent in the same system, acting on different parts of the molecule according to the conditions of the experiment.

So far most work has been done on the complex reactions between the haemoproteins catalase, peroxidase and methaemoglobin with H_2O_2, but free protohaematin, the prosthetic group of these haemoproteins, was not known to react with H_2O_2 in any way except when co-ordinated with pyridine. It has now been shown that the compound formed in this reaction is merely an oxidation product of haematin and is not a true haematin peroxide compound such as it is necessary to visualize in the biological systems mentioned. Urohaematin, however, probably by virtue of being fully dispersed in solution, forms a true compound with H_2O_2 and with ethyl hydroperoxide and so provides a model for a free haematin peroxide compound where the peroxide is co-ordinated with the iron while the porphyrin ring remains unaltered.

SUMMARY

1. Urohaematin is shown to combine with hydrogen peroxide and ethyl hydroperoxide in the presence of excess hydroxyl ion. For complete formation of the complex the total concentration of sodium hydroxide required was 0·7 N in the case of hydrogen peroxide and 1·5 N for ethyl hydroperoxide. The effect of hydrogen peroxide on urohaematin in other alkalis is discussed.

2. The urohaematin-peroxide complex has a characteristic absorption spectrum (α—547·5 mμ.; β—521 mμ.; γ—413 mμ.) which is of short duration. In the case of hydrogen peroxide the absorption bands of free urohaematin begin to replace it within a few seconds while with ethyl hydroperoxide the compound is reduced to dihydroxyl-haem.

3. In the urohaematin-peroxide compound one molecule of hydrogen peroxide is combined per molecule of urohaematin. The dissociation constant $K = 3{\cdot}34 \times 10^{-4}$ M at 20°.

4. It is suggested that dihydroxyl-urohaematin may be an essential intermediate for the subsequent formation of the urohaematin-peroxide compound, one molecule of hydrogen peroxide or ethyl hydroperoxide replacing one hydroxyl group on the urohaematin iron atom.

5. A protohaematin-peroxide compound has not so far been obtained with either hydrogen peroxide or ethyl hydroperoxide.

6. The pyridine parahaematins of both uro- and proto-haematin react with hydrogen peroxide and

ethyl hydroperoxide to give 'green compounds' with two-banded absorption spectra. The exact positions of these bands depend on the pH and the amount of peroxide added. In the case of protoparahaematin additional transient absorption bands are observed under certain conditions.

7. The 'green compound' given by pyridine uroparahaematin and ethyl hydroperoxide reduces spontaneously and reversibly to a compound which differs from pyridine urohaemochromogen in that the positions of its absorption bands are nearer the red and in that it does not combine with carbon monoxide. The green compounds given by pyridine protoparahaematin with hydrogen peroxide (pH 6·5) and ethyl hydroperoxide are probably of the same nature.

8. It is suggested that these 'green compounds' correspond to those given by pyridine protoparahaematin and hydrogen peroxide described by Haurowitz, but that they are oxidation products of the haematins and not compounds of the haematins with hydrogen peroxide.

9. Urohaematin-peroxide is so far the only example of a free haematin-peroxide compound where the porphyrin ring is unaltered.

I wish to thank Dr E. F. Hartree for a sample of ethyl hydroperoxide and Dr E. C. Slater for helpful discussions.

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A New Amino-acid Amide in the Groundnut Plant (*Arachis hypogaea*): Evidence of the Occurrence of γ-Methyleneglutamine and γ-Methyleneglutamic Acid

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A brief account of preliminary studies of a new metabolite detected in groundnut plants has already been given (Done & Fowden, 1951). The substance was isolated and found to be an unsaturated amino-acid amide. This earlier work is now described in more detail, together with subsequent investigations of the structure of the new compound.

The first material examined was the 'sap' produced when the turgid stems of young seedlings were incised between the cotyledons and the first leaf. About 20 μl. of liquid exuded from the cut surfaces. It was found by paper chromatography that the main ninhydrin-reacting component of the exudate gave an orange-brown spot ($R_F = 0{\cdot}66$ on chromatograms run in phenol-ammonia). The colour of the ninhydrin spot varied somewhat on different occasions, but always it was clearly outside the range of colours obtained with known α-amino-acids.

A large number of exudates produced in the manner described have been examined, and the new substance (I) has invariably been the dominant ninhydrin-reacting component. When amounts of the order of 20 μl. were chromatographed, much smaller spots due to asparagine, glutamine, aspartic acid, glutamic acid, alanine and other amino-acids sometimes appeared.

(I) has also been detected in homogenates of stems, leaves, hypocotyls and roots, but in these many other ninhydrin-reacting compounds were also present. The new compound could not be

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detected in seedlings less than 2 days after germination, but it occurred in all samples taken after the differentiation of the first leaf, e.g. in the exudate formed after the incision of the petiole of the fifteenth leaf of a main stem, and in the gynophores bearing immature fruits. The stem, root and hypocotyl of seedlings germinated and maintained in the dark for 12 days all contained (I). It was not detected in immature or ripe seeds, or in the hydrolysate of a commercial sample of arachin. Thus, the compound (I) appeared after germination of the seed and occurred in all the vegetative tissues examined.

A number of varieties of beans, peas and lupins, and the sprouts of potato tubers were examined, but no compound giving the type of ninhydrin reaction shown by (I) was observed on the chromatograms.

Preliminary examination of the chemical properties of (I)

The action of hydrochloric acid. Exudate was heated with 5N-hydrochloric acid at 105° for 16 hr. in a sealed tube; hydrochloric acid was removed by evaporation. (I) could not be detected on chromatograms, but an intense yellow-brown spot ($R_F = 0{\cdot}30$ in phenol-ammonia) appeared. Here again, the colour of the spot was quite distinct from those obtained with known α-amino-acids. Similar results were obtained after treatments with 5N-hydrochloric acid for 3 days at 105° and with N-hydrochloric acid for 3 hr. at 100°. Spots due to asparagine and glutamine occurred on chromatograms of the exudate, but the intensity of each of these was less than one-tenth of the intensity of the main spot due to (I). These amides were hydrolysed to the corresponding acids by the hydrochloric acid treatments.

When a sample of exudate containing 6·74 μg. N/μl. was treated with N-hydrochloric acid for 3 hr. at 100°, ammonia nitrogen equivalent to 2·7 μg./μl. was produced. The conditions of hydrolysis in this experiment were those used in the estimation of amide nitrogen (Borsook & Dubnoff, 1939), so the results could be explained by the hypothesis that amide I → acid II + NH_3. This was supported by the chromatographic evidence. The ratio of the R_F of (I) to that of (II) in phenol-ammonia was 2·2. The corresponding ratios for asparagine and glutamine are 2·5 and 2·0 respectively (Dent, 1948).

(I) and (II) both combined with copper as shown by the copper acetylacetonate test (Wieland & Fischer, 1948); thus the presence of α-amino nitrogen was indicated.

Autolysis. When homogenized leaf tissue was allowed to autolyse for 3 hr. at 37° chromatograms showed that (I) had been converted to (II). Both compounds occurred in ethanolic extracts of fresh plant 'tops'.

Isolation of compound (I). (I) was retained when diluted exudates were run through a column of Zeo-Karb 215 (Partridge & Westall, 1949) and the compound was isolated after elution with ammonia solution. It was readily soluble in cold water and formed colourless plates on crystallization from aqueous ethanol. Elementary analysis gave an empirical formula $C_6H_{10}O_3N_2$. Half the nitrogen was released as ammonia by treatment with N-sodium hydroxide for 24 hr. at room temperature, and by treatment with N-hydrochloric acid for 3 hr. at 100°.

Isolation of compound (II). De-Acidite B (Partridge & Brimley, 1949) retained compound (II) when an autolysate of the plant 'tops' was run through a column of the material. The new acid was eluted by acetic acid followed by dilute hydrochloric acid, and it was isolated by recrystallization of the solid left after evaporation of fractions of the effluent from the column. The compound was only slightly soluble in cold water but much more soluble in hot water. It was therefore convenient to crystallize the compound from hot water. Elementary analysis gave an empirical formula $C_6H_9O_4N$. All the nitrogen was amino nitrogen as measured by the Van Slyke nitrous acid method. The equivalent weight found by titration with sodium hydroxide was 158 (formula weight 159). No optical activity was detected in an aqueous solution of the sodium salt.

The structure of the new compounds

The analyses of the new compounds confirmed the chromatographic evidence that (II) was an α-amino-acid derived from (I) by deamidation.

The presence of a double bond. Both compounds decolorized a solution of potassium permanganate at room temperature. Treatment with ozone gave a substance which could not be distinguished from aspartic acid by paper chromatography. The new amide therefore appears to resemble asparagine and glutamine in that the amide group is not attached to the α-carbon atom.

The product of the action of ozone on (II) was isolated and shown to be aspartic acid by exhaustive methylation to fumaric acid (Braunstein, Nemschinskaya & Vilenkina, 1947).

When (II) was hydrogenated in the presence of palladium-on-carbon catalyst, the amount of gas absorbed corresponded to that required for a reaction $C_6H_9O_4N \rightarrow C_6H_{11}O_4N$.

The reduced form of (II), compound (III). Elementary analysis of (III) gave the empirical formula $C_6H_{11}O_4N$. The compound formed a purple spot with ninhydrin at R_F 0·42 on chromatograms run in phenol-ammonia. The aqueous solution showed no optical rotation.

Since aspartic acid was produced by the action of ozone on (II), it seemed likely that the compound

contained $COOH.CH(NH_2)CH_2C=$, and since a titratable carboxyl group has also been detected, possible formulae were

$$COOH.CH(NH_2).CH_2.CH=CH.COOH$$

and

$$COOH.CH(NH_2).CH_2.C(=CH_2).COOH.$$

The corresponding saturated compounds are α-aminoadipic and γ-methylglutamic acids respectively.

Substance (III) differed from DL-α-aminoadipic acid in its solubility and melting point and in its position on paper chromatograms. α-Aminoadipic acid is converted to ornithine by hydrazoic acid (Adamson, 1939). However, treatment of (III) with hydrazoic acid gave a substance which could be separated from ornithine on chromatograms run in aqueous *n*-butanol-acetic acid mixture (Partridge, 1948). It was therefore evident that (III) was not DL-α-aminoadipic acid.

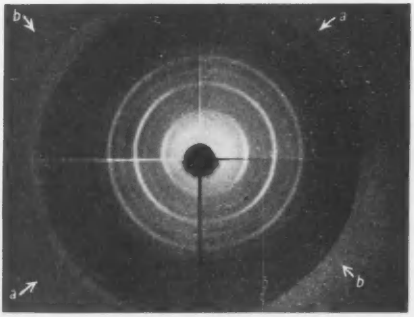
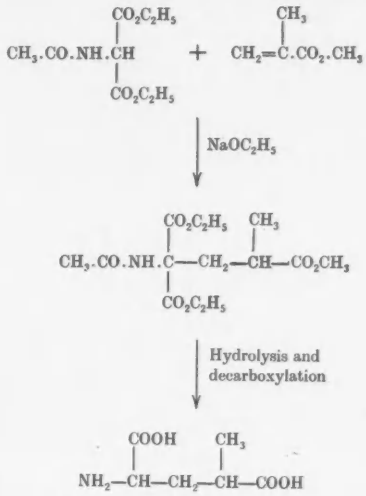


Fig. 1. X-ray powder photographs of (*a*) compound (III) and (*b*) synthetic γ-methylglutamic acid. Cu (Ni filtered) $K\alpha$ radiation; specimen to film distance 5 cm.

On the other hand, it had been found that the melting point of (III) was low compared with those of most of the known α-amino-acids, and pyrrole derivatives had been detected in the vapour formed when (III) was fused with zinc dust. These facts suggested that the compound contained an α-amino and a γ-carboxyl group, and it was considered that (III) might be γ-methylglutamic acid.

Synthesis of γ-methylglutamic acid. Glutamic acid has been synthesized by Michael condensations: methyl acrylate and ethyl phthalimidomalonate were employed by Marvel & Stoddard (1938), and methyl acrylate and ethyl acetaminomalonate by Snyder, Shekelton & Lewis (1945).

Evidence of the synthesis of (III) by condensation of methyl methacrylate with ethyl phthalimidomalonate was obtained. However, better results were obtained by the reaction between methyl methacrylate and ethyl acetaminomalonate:



Elementary analysis of the synthetic material gave the empirical formula $C_6H_{11}O_4N$; when fused with zinc dust it gave a positive pine-splinter test for pyrrole derivatives.

The position of the synthetic compound on paper chromatograms coincided with that of the natural acid (III). Both substances melted at 154–155° and the melting point of a mixture was unchanged.

Prof. Kathleen Lonsdale, F.R.S., very kindly arranged for X-ray powder photographs of the natural and synthetic acids to be prepared (Fig. 1). She reported that the two samples gave identical results.

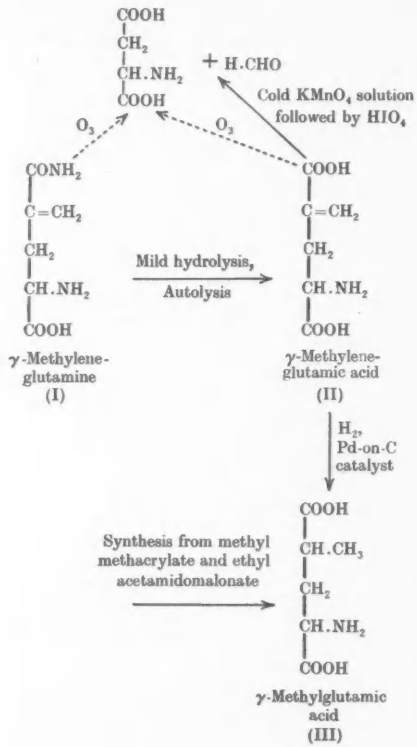
Oxidative degradation of (II). The synthesis of (III) had provided good evidence of the carbon skeleton of the new compounds. The possible position of the double bond had been indicated by the formation of aspartic acid by treatment of the unsaturated compounds with ozone. However, the yield of aspartic acid was low, and no non-nitrogenous fragment had been isolated. Accordingly, further experiments were made on the oxidative degradation of (II).

The compound reacted almost completely with an approximately equimolecular amount of potassium permanganate in ice-cold aqueous solution. Further oxidation with periodic acid gave, on different occasions, yields of formaldehyde (dimedone derivative) of 41, 27 and 25% (mol. basis). The main ninhydrin-reacting component resulting from permanganate-periodate oxidation could not be separated from aspartic acid on chromatograms run in phenol-ammonia, butanol-acetic acid

(Partridge, 1948) or collidine-lutidine solvents. Evidence that aspartic acid was formed in 32% yield was obtained by a quantitative chromatographic method (Fowden, 1951).

Exhaustive methylation (Braunstein *et al.* 1947) of the product of oxidative degradation after removal of formaldehyde gave fumaric acid, corresponding to a yield of 29% of aspartic acid.

Provisional structures of the new compounds are included in the following scheme, which also summarizes the main reactions described:



EXPERIMENTAL

All melting points are uncorrected.

Isolation of compound (I). Young plants (3000–4000) were grown in boxes containing either garden soil or gravel and nutrient salts. Exudates were obtained by cutting the main stems when four to eight leaves had developed. They were collected in capillary tubes, and stored in the refrigerator in 2–4 ml. batches. The precipitate which formed was removed by centrifugation. A total of approx. 40 ml. of material was obtained. Preliminary attempts to concentrate or crystallize (I) by extraction of dried exudate with ethanol and by other methods were not successful.

A column of Zeo-Karb 215 was prepared (Partridge & Westall, 1949), 15 cm. in length and 0·9 cm. in diameter. The exudate material was diluted with 250 ml. of water and passed through the column at the rate of 1 drop in approx. 4 sec. The resin darkened progressively from the top as the mixture passed through the column, until finally only a few mm. at the bottom remained unchanged. Distilled water (1 l.) was used to wash the column and then a solution of 0·15 N-NH_3 was run through. Eighteen fractions of vol. 10–15 ml. were collected.

A light-coloured band moved down the column during elution. This was apparently due to (I), since when it 'broke through' fraction 9, 5 μl. of the effluent gave a strong yellow to red colour when treated with ninhydrin on filter paper. Chromatograms of 50 μl. amounts of the eluates were run in phenol-NH_3. The results are given in Table 1.

Table 1. *Elution of compound (I) from Zeo-Karb 215 with dilute ammonia solution*

(For details, see text.)

Fractions (serial nos.)	Appearance of filter-paper chromatogram (Phenol-NH_3-water)
1–8	Faint (I) spot
9	Fairly strong (I) spot
10–12	(I) spot about 10 times as strong as that in fraction 9. Faint pink spot at origin
13–17	(I) spot as in preceding fractions. Faint purple streaks before and after (I)
18	(I) spot about half as strong as that of fraction 9

Fractions 1–7 were discarded. Fractions 8–17 were evaporated to dryness over H_2SO_4 in a vacuum desiccator. 190 mg. of residue were obtained which yielded 120 mg. of material in flat colourless crystals after two crystallizations from aqueous ethanol. (Found: C, 46·0; H, 6·5; N (Dumas), 17·3. $C_6H_{10}O_5N_2$ requires C, 45·6; H, 6·3; N, 17·7%.) When heated in a sealed melting-point tube at the rate of about 3°/min., signs of decomposition appeared at 173° and increased until 182° had been reached. No melting or further change occurred up to 260°. The tube was cut open at both ends, and the vapour inside blown over the surface of a moist litmus paper which then gave a strong alkaline reaction.

(I) dissolved very readily in water, and the solution gave a slightly acidic reaction with bromothymol blue.

Estimation of amide nitrogen. The microdiffusion method of Dekker, Stone & Fruton (1949) was used, adapted to a small diffusion unit made of paraffin wax.

A solution (50 μl.) made by dissolving 2·223 mg. of (I) in 1 ml. of water was placed in one depression in the wax. 2 N-NaOH (50 μl.) was placed in a second depression, and 3 N-H_2SO_4 (100 μl.) in a third. The unit was covered by a glass plate and sealed by melting a little wax at the edges of the glass. The alkali was then shaken into the test solution, and the unit left at room temperature for 30 hr. The acid was removed to a test tube, and the alkaline residue digested with nitrogen-free H_2SO_4 and a selenium catalyst. Nitrogen in the mixtures and in a digest of the original solution was estimated colorimetrically after nesslerization. (Found: diffused N, 9·3; residual N, 9·1; total N, 18·2%.)

Following Borsook & Dubnoff (1939), who determined amide N by hydrolysis in N-acid, 1 ml. of solution as used in

the preceding experiment was heated for 3 hr. with 0·5 ml. 3N-H_2SO_4 at 100°. NH_3-N in the hydrolysate, as determined by nesslerization, was equivalent to 8·7% of (I).

Treatment with ozone. 5 mg. of (I) dissolved in water were treated with ozone. When the solution was concentrated in a vacuum desiccator, extensive decomposition occurred with the formation of carbonaceous matter. The main ninhydrin-reacting component gave a purple spot which coincided in position on paper chromatograms with that due to aspartic acid. It could not be separated from added aspartic acid on two-dimensional or long-developed one-dimensional chromatograms in aqueous phenol-NH_3 and *n*-butanol-acetic acid (Partridge, 1948).

The isolation of compound (II). The tops of the young plants from which the exudates had been obtained were broken up in water in a blender and then incubated overnight at 37° in the presence of a little thymol. The autolysates were filtered through muslin and stored in the refrigerator. The autolysates were pooled, charcoal was added and the mixture stirred for 30 min. at room temperature and filtered. The final volume was about 10 l.

The liquid was passed through a column of Zeo-Karb 215, which adsorbed all the ninhydrin-reacting substances. The column was washed with several litres of distilled water, and then a solution of 0·15N-NH_3 was run through. The effluent was evaporated under reduced pressure until the volume was 1·8 l., and then extracted several times with ether to remove fat. Dissolved ether was removed by aeration, and the liquid again treated with charcoal at room temperature and filtered.

A column of De-Acidite B was prepared (Partridge & Brimley, 1949) measuring 40 × 2·4 cm. The liquid was run through at the rate of 5–6 ml./min., and the effluent collected in fractions of approx. 250 ml.

Chromatograms showed that the column had removed the acidic components (aspartic and glutamic acids and compound II) from the first 1·1 l. of liquid. The later fractions (about 650 ml.) contained the acidic as well as other amino-acids.

The column was washed with 1·8 l. of 0·1N-acetic acid followed by 500 ml. of 0·25N-HCl, at the rate of about 100 ml./hr. Fractions were collected at 50–60 min. intervals

Chromatograms of fractions 1–6 showed glutamic acid and only traces of (II), so they were discarded. Pooled fractions were evaporated by distillation at reduced pressure, and residual water was removed by drying over NaOH in a vacuum desiccator.

Fractional crystallization of the products from water yielded 1·063 g. of crystals which gave only spot (II) on chromatograms, and a number of samples of total weight 2·680 g. which were found to contain slight traces of glutamic acid. The former batch was used for analytical and other quantitative work. The other samples were used for qualitative examinations, and, after further crystallizations, for the preparation of derivatives.

Some properties of compound (II). When heated in a melting-point tube, decomposition commenced at 196°, after which the substance changed in colour through yellow to dark brown at 325°. With more rapid heating (i.e. greater than 2°/min.) the first change occurred at 204°, when the surface of the substance became 'moist'.

The acid had a low solubility in water. No optical activity was detected when 96·3 mg. were made neutral to phenolphthalein with NaOH in water (final vol. 1·82 ml.) and observed in a 1 dm. polarimeter tube. (Found: C, 44·1; H, 5·7; N, 8·6; amino N (Van Slyke nitrous acid method, 4 min. reaction), 8·4. $C_6H_9O_4N$ requires C, 45·2; H, 5·7; N, 8·8%.) When 164 mg. of (II) was titrated with 0·735N-NaOH to a faint pink to phenolphthalein, the equiv. wt. was found to be 158 (formula wt. 159). CO_2 was evolved when (II) was dissolved in 10% (w/v) Na_2CO_3 solution.

Treatment with ozone. 100 mg. of (II) was suspended in 7 ml. of water and ozonized oxygen was passed through for 45 min. The product of the treatment was at first a clear solution, but extensive decomposition occurred when it was allowed to stand overnight. The main ninhydrin-reacting component found on chromatograms coincided in position with aspartic acid.

After removal of the charred material by filtration the solution was boiled with $CuCO_3$. The excess $CuCO_3$ was removed by filtration, and, on cooling, a copper compound of low solubility in water crystallized out. Copper was removed from this by H_2S, and 11·1 mg. of a white crystalline solid were obtained by crystallization from aqueous ethanol. This was shown to be aspartic acid by exhaustive methylation to fumaric acid (Braunstein *et al.* 1947). The product of exhaustive methylation, isolated from the reaction mixture with ether by means of a small continuous extraction apparatus, melted at 295° in a sealed tube. The melting point was unchanged when the product was mixed with an authentic sample of fumaric acid.

Oxidation with permanganate. $KMnO_4$ solution (1%, w/v) was added to about 1 ml. of a saturated solution of (II), until there was a slight permanent pink colour. This colour was discharged by adding 1 drop more of the solution of (II). The mixture was filtered and electrolytically desalted (Consden, Gordon & Martin, 1947).

Chromatograms run with aqueous *n*-butanol-acetic acid mixture showed a prominent spot which coincided in position with aspartic acid. Two other spots were visible: a well marked spot of lower R_F, and a faint spot of higher R_F than the aspartic acid.

In another experiment 96 mg. of (II) were dissolved in 4 ml. 10% (w/v) Na_2CO_3 solution. Finely powdered $KMnO_4$ was added until a slight excess could be detected. A few mg. more of (II) were then added. The mixture was warmed to about 60° for a few minutes, when coagulation of MnO_2 commenced. It was then filtered, and the precipitate washed. A portion of the filtrate was desalted and chromatograms showed a single spot coincident in position with aspartic acid. However, the latter could not be isolated as the Cu salt, presumably because of the high concentration of electrolytes in the mixture. Later, oxalic acid was isolated from residues from this experiment through the Ca salt (melting point of the di-*p*-toluidide 264°, unchanged when mixed with an authentic sample).

Oxidation with cold aqueous permanganate was studied in more detail later.

Reduction of compound (II) to compound (III) and investigation of the product. In one experiment, 28·9 ml. of H_2 were absorbed by 180 mg. of (II) suspended in 10 ml. water with 50 mg. palladium-on-carbon catalyst. (The theoretical amount for a reaction $C_6H_9O_4N \rightarrow C_6H_{11}O_4N$ under the same experimental conditions was 27·8 ml.)

After removal of the catalyst, the solution was evaporated in a vacuum desiccator. Solid separated when 1–2 ml. of solution remained. This was dissolved by addition of the minimum of water. Acetone was added and the mixture left overnight in the refrigerator. 154 mg. of product were obtained after filtration. (Found: C, 44·7; H, 6·8; N, 9·1. $C_6H_{11}O_4N$ requires C, 44·7; H, 6·8; N, 8·7%.) Equiv. wt. by

titration with 0·103 N-NaOH was 159 (equiv. wt. of monobasic $C_8H_{11}O_4N$, 161). Melting points varied from 154–165°, apparently depending on the rate of heating and the amount of material used.

2–3 mg. of (III) were shaken with conc. H_2SO_4 and excess of N_3H in $CHCl_3$ at 43–46° (Adamson, 1939). A sample of the reaction product was desalted and on paper chromatography the main ninhydrin-reacting component of the solution had a higher R_F value than ornithine in aqueous *n*-butanol-acetic acid mixture, being clearly separated from added ornithine.

Synthesis of γ-methylglutamic acid. Preliminary experiments were based on the glutamic acid synthesis of Marvel & Stoddard (1938), methyl methacrylate (containing quinol to inhibit polymerization) being used in place of the methyl acrylate. Methyl methacrylate and diethyl phthalimidomalonate were refluxed in absolute ethanol in which small amounts of sodium had been dissolved. Volatile components were removed after different periods of refluxing, and samples of the residues were hydrolysed overnight with 20% (w/v) HCl in sealed tubes at 105°. Chromatograms run in aqueous *n*-butanol-acetic acid showed the presence of glycine (presumably from free diethyl phthalimidomalonate) and another component coincident in position with compound (III) and presumably γ-methylglutamic acid. The glycine and phthalic acid also present could not be separated from this by crystallization from aqueous ethanol or aqueous acetone. A copper salt of low solubility in water was obtained from the hydrolysis product, which, after removal of the copper by H_2S, was found to yield only a trace of glycine on the chromatograms run in butanol-acetic acid solvent, together with an intense spot coincident in position with (III). 1·83 g. of the copper salt were obtained from 23·8 g. of diethyl phthalimidomalonate. However, preliminary experiments indicated that better results could be obtained by a different procedure.

Snyder *et al.* (1945) synthesized DL-glutamic acid from methyl acrylate and diethyl acetaminomalonate. In the experiment to be described, the conditions were similar to those in the above-mentioned synthesis except that sulphur was added to inhibit polymerization of methyl methacrylate and that an intermediate was isolated and recrystallized before hydrolysis.

Sodium (120 mg., 0·0052 g.atom) was dissolved in approx. 20 ml. ethanol in a 200 ml. flask. 100 mg. sulphur and 10·85 g. (0·05 mole) ethyl acetaminomalonate were added. An attachment bearing a mechanical stirrer, a reflux condenser, and a dropping funnel was fixed to the flask. The contents were refluxed and 10 g. (0·1 mole) of methyl methacrylate were added in drops during 4 hr. Refluxing was continued for a further hour.

The reaction mixture yielded 7·34 g. of colourless crystals after three crystallizations from ethanol. Melting point 109·5°, unchanged on recrystallization. (Found: N (Kjeldahl), 5·7, 5·8%.) This substance was not, apparently, the primary condensation product of methyl methacrylate and ethyl acetaminomalonate, the N content of which would be 4·6%. A small sample was hydrolysed overnight by 20% (w/v) HCl in a sealed tube at 105°, and chromatograms run with *n*-butanol-acetic acid showed a spot coincident in position with (III), and no glycine. 2·78 g. of the intermediate was hydrolysed by refluxing in 20% (w/v) HCl, more HCl being added from time to time to replace the acid which had been used in the reaction.

The hydrolysate was evaporated almost to dryness at reduced pressure. The process was repeated three times after addition of 5 ml. amounts of water. The product was then dissolved in 50 ml. of water. Residual HCl was removed by two extractions of the solution with excess of a 5% (w/v) solution of trioctylamine in $CHCl_3$ (Lester Smith & Page, 1948). The aqueous layer was washed twice with $CHCl_3$ and evaporated in a vacuum desiccator until solid appeared. A further batch of the intermediate (3·62 g.) was hydrolysed and the acid-free product combined with that from the first batch. The crude product was dissolved in the minimum of water. Acetone was added, and the mixture cooled in crushed ice and left overnight in the refrigerator. The resulting solid was filtered off, dissolved in the minimum amount of water, and again treated with acetone, cooled and left overnight in the refrigerator. 2·82 g. crystalline solid were obtained (45% yield, based on the diethyl acetaminomalonate). (Found: C, 44·9; H, 6·8; N, 8·5. $C_8H_{11}O_4N$ requires C, 44·7; H, 6·8; N, 8·7%.)

Oxidative degradation of acid (II). 1·4 ml. of 0·735 N-NaOH were added to 161 mg. of acid (II). The solution thus obtained gave a light-green colour with bromothymol blue. A solution of 158 mg. of $KMnO_4$ in 10 ml. water was added in drops over 45 min. The reaction was carried out in a cold room and the temperature of the mixture maintained at 5–6°. After the reaction, the mixture was allowed to stand for 30 min. at laboratory temperature. It was then warmed to 30–35° in a water bath for a few minutes to coagulate the MnO_2. The precipitate was removed by centrifugation and washed with four lots of water (total vol. 10 ml.). A sample (0·5 ml.) was removed.

A faint turbidity developed when aqueous dimedone solution was added to part of the sample. The remainder of the sample was desalted. Chromatograms run with phenol-NH_3 and *n*-butanol-acetic acid solvents showed that the main ninhydrin-reacting component had a lower R_F than aspartic acid. Smaller amounts of aspartic acid, compound (II) and compound (III) were also present. (The compound (III) had probably been formed by reduction of compound (II) in the desalter.)

0·75 g. of $HIO_4.2H_2O$ was added to the combined supernatants. A slight red precipitate formed. This was removed after 1 hr. by centrifugation and the mixture was allowed to stand for a further 30 min. CO_2 was slowly evolved during the periodate reaction. Periodate was precipitated as the Ba salt by addition of $Ba(OH)_2$ in slight excess. The precipitate was washed on the centrifuge, and the combined supernatants made slightly acid with H_2SO_4 and filtered.

The filtrate was distilled and the distillate collected in an aqueous solution containing approx. 260 mg. of dimedone. 71 mg. of a dimedone derivative were obtained. Its melting point was 189°, unchanged when mixed with an authentic sample of the formaldehyde derivative (yield corresponding to 25% of formaldehyde, mol. basis).

A sample of residue from the distillation was desalted. Chromatograms showed a heavy spot corresponding in its position with aspartic acid. The spot could not be separated from that due to added aspartic acid on further chromatograms run in phenol-NH_3, *n*-butanol-acetic acid, or collidine-lutidine solvents. A desalted sample of the residue from the distillation was analysed by a quantitative chromatographic method (Fowden, 1951). The amount of amino N in the spot referred to above was found to correspond to a yield of 32% (mol. basis) from the amount in the original substance. A similar experiment gave 27% of formaldehyde.

377 mg. of (II) were oxidized in a third experiment. 370 mg. of $KMnO_4$ and 1·3 g. of $HIO_4.2H_2O$ were used. After the treatment with $Ba(OH)_2$, 330 mg. of dimedone dissolved in hot water were added. The mixture was left overnight at room temperature, and the resulting precipitate filtered off and washed with cold water. 301 mg. of formaldehyde derivative were obtained (mixed m.p. 189°, yield 41%). The filtrate was evaporated to small bulk, filtered, and treated with dimethyl sulphate and 33% (w/v) NaOH (Braunstein *et al.* 1947). 81 mg. of fumaric acid (29% yield) were obtained after continuous ether extraction (m.p. 292° in a sealed tube; 295° after crystallization from water, unchanged on admixture of an authentic sample).

DISCUSSION

The oxidative degradation of (II) and the synthesis of its reduced form have provided evidence on the basis of which the provisional structure

$$COOH.CH(NH_2).CH_2.C(=CH_2)COOH$$

(γ-methyleneglutamic acid) is proposed. The identification of aspartic acid by paper chromatography after treatment of the amide with ozone indicates that the amide is

$$COOH.CH(NH_2)CH_2.C(=CH_2).CONH_2$$

(γ-methyleneglutamine).

Steward & Thompson (1950) presented a synopsis of the results of paper-chromatographic examination of ethanol extracts of various plant materials. They suggested that amides may occur among a group of acid-labile substances detected. However, although asparagine was isolated by Vauquelin & Robiquet (1806) and glutamine by Schulze & Bossard (1883), no similar amino-acid amide has been isolated before the present investigation.

The detection and isolation of the new amino-acid amide (tentatively recognized as γ-methyleneglutamine) and the corresponding acid were made possible by the techniques of paper chromatography. Among other favourable circumstances were: the high concentration of the amide in the exudates, the distinctive ninhydrin reaction of the amide and acid, the low solubility of the acid in water, and the fact that autolysis caused only partial deamidation of asparagine and glutamine, but apparently almost complete deamidation of γ-methyleneglutamine.

The autolysate from which γ-methyleneglutamic acid was isolated contained a substance (IV) which gave a yellow-brown spot at R_F 0·85 in phenol–ammonia. Traces of the same substance were found in the end residues from the fractional crystallizations of the acid, along with a number of other amino-acids. (It is likely that non-acidic amino-acids were retained to some extent on the De-Acidite B and were eluted by the ammonia.) A small quantity of (IV) was isolated from chromatograms of these residues by elution of the appropriate parts of the paper. After treatment with ozone, the product contained a substance which could not be distinguished from β-alanine on chromatograms run in three different solvents. β-Alanine could be produced from α-methylene-γ-aminobutyric acid:

$$COOH.\overset{\overset{CH_2}{\|}}{C}.CH_2.CH_2.NH_2 \xrightarrow{O_3} COOH.CH_2.CH_2.NH_2$$

γ-Aminobutyric acid and β-alanine, the ω-amino-acids related to glutamic and aspartic acids respectively, are now known to be widely distributed among plants. The possible existence of an ω-amino-acid related to γ-methyleneglutamic acid is therefore of considerable interest.

Compounds (I), (II) and (IV) were converted, in the electrolytic cell of the desalter, to substances which gave purple ninhydrin spots. After such treatment, the substance formed from (II) could not be distinguished from compound (III) by paper-chromatographic methods. The R_F's of the substances formed from (I) and (IV) differed only slightly from those of the original compounds. Apparently compounds (I) and (IV) are also reduced to substances which give a purple colour with ninhydrin. None of the reduced forms has been detected in ethanolic extracts of the plant material.

Application of modern methods to amino-acid analysis and experiments using isotopic tracers are providing increasing evidence that glutamine, asparagine, glutamic acid and aspartic acid are very active metabolites. Thus interesting results may be obtained from further work on the biochemistry of the new type of amide and the corresponding acid described in this paper.

SUMMARY

1. An unsaturated amino-acid amide and the corresponding dicarboxylic amino-acid have been isolated from vegetative material of groundnut plants (*Arachis hypogaea*). The acid showed no optical activity as its sodium salt in water.

2. The provisional structures

$$COOH.CH(NH_2)CH_2.C(=CH_2)CONH_2$$

(γ-methyleneglutamine) and

$$COOH.CH(NH_2)CH_2.C(=CH_2)COOH$$

(γ-methyleneglutamic acid) have been proposed.

3. The dicarboxylic amino-acid was reduced and the product agreed in properties with a synthetic preparation of γ-methylglutamic acid.

4. The new compounds appear to be normal metabolites of the plants, but the reduced forms were not detected in ethanolic extracts of fresh material.

5. Evidence was obtained that a third unsaturated amino compound was present in the autolysed material.

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The Carotenoids of the Berries of *Lonicera japonica*

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Although a great deal is known concerning the distribution and occurrence of carotenoids in fruit (see Karrer & Jucker, 1949; Goodwin, 1952*b*), there are still many gaps in our knowledge; these will have to be filled if a rationale of carotenoid distribution in fruit is eventually to be achieved. Furthermore, studies on the minor carotenoid components of fruit may well throw considerable light on the mode of biogenesis of carotenoids. Only one such investigation on tomatoes has been carried out (Porter & Lincoln, 1950); the results permitted the authors to suggest a working hypothesis for carotenogenesis in this fruit.

No precise information is available on the carotenoids of the berries of the family Caprifoliaceae, although early workers had indicated the presence of lipochromes (carotenoids) in *Lonicera tartarica* (Schimper, 1885), *L. xylosteum* (Schimper, 1885; Molisch, 1896; Kohl, 1902; Nowak & Zellner, 1921); *Sambucus nigra* (Nowak & Zellner, 1921), *Viburnum opulis* and *V. lantana* (Wisselingh, 1914; Nowak & Zellner, 1921; Kryz, 1919). When a small crop of ripe berries of a cultivated climbing honeysuckle (*Lonicera japonica*) became available an investigation was undertaken into the nature of the carotenoids present, with special emphasis on the minor components.

EXPERIMENTAL

Materials. The fully ripened berries were obtained from a garden in north-west Cheshire. Two portions of about 200 g. each were examined separately with identical results.

Extraction and separation of the carotenoids

The fresh berries were ground to a fine powder with Na_2SO_4 and the powder extracted with successive portions of diethyl ether (freshly distilled over reduced iron) until no further colour was extracted. The combined ether extracts were taken to dryness *in vacuo* at room temperature and the lipids saponified by adding to the residue 1 ml. of 60% (w/v) aqueous KOH and 5 ml. of ethanol, mixing and allowing to stand overnight at room temperature. The unsaponifiable matter was extracted into ether as described by Goodwin & Morton (1946) and the ether was removed *in vacuo*; the residue was dissolved in a small volume of light petroleum (b.p. 40–60°) and examined chromatographically. Petroleum, b.p. 40–60°, was used throughout this investigation.

Separation 1. A preliminary separation of the pigment mixture was first carried out on alumina (Spence, Grade 'O') deactivated with methanol (Goodwin, 1952*a*) using light petroleum containing 10% (v/v) of ether as developer. The resulting chromatogram is described in Table 1.

Separation 2. Fraction 1 (Table 1), which eventually percolates through the column, was collected in the filtrate, the mixed solvents removed *in vacuo* and the residue redissolved in light petroleum and chromatographed on activated alumina (Spence, Grade 'O'). Five fractions were obtained as recorded in Table 2.

Separation 3. Zone 6 (Table 1) was eluted with ethanol. The ethanol was removed *in vacuo* at 30° and the residue dissolved in a few drops of ether and made to approx. 10 ml. with light petroleum. This treatment is necessary because this fraction will not dissolve directly in light petroleum. Chromatography was carried out on a column of $ZnCO_3$ using benzene as developer. Four zones were obtained, two of which eventually moved down the column to be collected

in the filtrate (Table 3). On treatment of the column with benzene containing 5% (v/v) of ethanol, fraction 6*C* moved off the column and fraction 6*D*, originally adsorbed at the top, separated into two zones which moved slowly down the column. The faster moving pigment was designated 6*DA* and the slower moving one 6*DB*.

Table 1. *The first separation of* Lonicera *carotenoids on a column of deactivated alumina (Goodwin, 1952a), using light petroleum containing* 10% *(v/v) ether as developer*

(The zones are numbered in order of increasing adsorptive power.)

Zone no.	Description	Absorption spectrum maximum (solvent, light petroleum) (mμ.)
1	Orange, diffuse; greenish blue fluorescence*	—
2	Narrow, orange-khaki	490, 460, 440
3	Pink, slight blue fluorescence*	471, 445
4	Orange	479, 450
5	Trace of khaki	449
6	Orange-red, major fraction: strongly adsorbed	452

* In ultraviolet light.

Table 2. *The separation of fraction* 1 *(Table* 1*) on a column of activated alumina (Spence, Grade 'O'), using light petroleum containing* 20% *(v/v) ether as developer*

(The zones are numbered in order of increasing adsorptive power.)

Zone no.	Description	Absorption spectrum maximum (solvent, light petroleum) (mμ.)
1*A*	Trace of blue-green fluorescence*	348, 367
1*B*	Lemon-yellow, diffuse, slight blue fluorescence*	399, 425
1*C*	Colourless, bright green-blue fluorescence*	531, 348, 367
1*D*	Diffuse-orange: major zone	450, 474
1*E*	Small, narrow, pale lemon	381, 401, 427†

* In ultraviolet light. † In ether.

Table 3. *The separation of fraction* 6 *(Table* 1*) on a column of* $ZnCO_3$*, using benzene as developer*

(The zones are numbered in order of increasing adsorptive power.)

Zone no.	Description	Absorption spectrum maximum (mμ.)
6*A*	Orange-red, major fraction	464, 491*
6*B*	Small zone, khaki	438, 463*
6*C*	Small brick-red zone	453, 479†
6*D*	Lemon-yellow	429 (broad)†

* Solvent benzene. † Solvent ethanol.

Examination of the fractions

Fractions 2, 3 *(Table* 1*)*, 1*C*, 1*D*, 1*E (Table* 2*)*, 6*A*, 6*C*, 6*DB* (Table 3). Examination of these fractions, after further chromatographic purification on appropriately activated alumina, indicated that in all probability they were γ-carotene (2), lycopene (3), phytofluene (1*C*), β-carotene (1*D*), ζ-carotene (1*E*), cryptoxanthin (6*A*), zeaxanthin (6*C*) and auroxanthin (6*DB*). In each case, except the last, the berry carotenoid was compared chromatographically and spectroscopically with an authentic and chromatographically homogeneous sample of the corresponding pigment. Samples of all the carotenes were obtained from the fungus *Phycomyces blakesleeanus* (Goodwin, 1952*a*). Cryptoxanthin and zeaxanthin were obtained from a commercial sample of maize meal.

In the case of fraction 6*DB* no authentic specimen of auroxanthin was available, but, as demonstrated later, the properties of this pigment (Karrer & Rutschmann, 1942) are so distinctive, that it is virtually impossible to confuse it with any other carotenoid.

Fractions 1*A*, 1*B (Table* 2*)*, 6*B and* 6*DA (Table* 3*)*. 1*A* appears to be closely related to phytofluene, whilst 1*B* is considered to be a carotene not previously described, it is proposed to call it η-carotene. Fraction 4 appears to be very similar to the unidentified pigment reported in human milk (Kon & Mawson, 1950), whilst fractions 5, 6*B* and 6*DA* remain unidentified.

Quantitative experiment

In order to obtain information on the relative amounts of the constituent pigments present in the berries, in one experiment the fractions obtained, as described above, were dissolved in known volumes of light petroleum and the extinctions of the solutions measured at the wavelengths of maximal absorption of the pigments concerned. Using the

Table 4. *The wavelengths and* $E^{1\%}_{1\,cm.}$ *values (in light petroleum) used for quantitative determination of* Lonicera *carotenoids*

Carotenoid	Wavelength (mμ.)	$E^{1\%}_{1\,cm.}$	Reference
Phytofluene	348	1200	Porter & Lincoln (1950)
β-Carotene	450	2580	Zscheile *et al.* (1942)
ζ-Carotene	422	2500	Porter & Lincoln (1950)
γ-Carotene	459	2760*	Zechmeister (1944)
Lycopene	469	3460*	Zechmeister (1944)
Cryptoxanthin	451	2460	Zscheile *et al.* (1942)
Zeaxanthin	452	2480	Zscheile *et al.* (1942)
Auroxanthin	425	1780*	Karrer & Jucker (1945)

* Calculated from graphs.

extinction values of the pigments recorded in Table 4, the amount of each pigment could be calculated. For this present purpose the $E^{1\%}_{1\,cm.}$ (max.) values for the new carotene and the unidentified xanthophylls were assumed to be 2500.

RESULTS

A. *The carotenes*

The following carotenes were identified unequivocally: phytofluene, β-carotene, ζ-carotene, lycopene and γ-carotene. They were chromatographically indistinguishable from authentic specimens of the corresponding compounds, and the shape and position of their absorption bands in the region 380–500 mμ. (320–400 mμ. in the case of phytofluene) were also identical with those of the known polyenes. Full details have recently been given of the chromatographic and spectral properties of these pigments (Goodwin, 1952*a*), and further elaboration is unnecessary here.

Fraction 1*A* (*Table* 2). This fraction, which only occurred in very small amounts, had an absorption spectrum very similar to that of phytofluene, but appeared to be rather less strongly adsorbed on alumina than this polyene. Insufficient material was available to examine it in any detail, but these preliminary observations do suggest that it is quite distinct from phytofluene.

Fraction 1*B* (*Table* 2). This fraction runs down an alumina column as a diffuse dull orange band just ahead of phytofluene and α-carotene. When these chromatographic properties are considered in conjunction with its spectral properties (Fig. 1), there is no doubt that this is a new carotene. The only carotene with similar chromatographic behaviour is ε-carotene obtained by Strain & Manning (1943) from the diatoms *Nitzschia closterium* and *Navicula torquatum*. A comparison of the spectrum of ε-carotene and that of the *Lonicera* carotene given in Fig. 1 shows that they are quite distinct. The only carotene already reported with an absorption spectrum at all similar to that of the pigment in fraction 1*B* is ζ-carotene (Porter & Lincoln, 1950). This pigment is, however, easily separable from the *Lonicera* carotene on a chromatogram on alumina (4 parts activated; 1 part deactivated); ζ-carotene is much more strongly adsorbed, for it forms a zone above β-carotene whilst the new carotene travels down the column well in front of β- (and even α-) carotene. Furthermore, ζ-carotene (1*E*) and the new carotene occur together in *Lonicera*, and are easily separable. The uniqueness of this carotene being so apparent, it is suggested that it be named η-carotene. This nomenclature follows the recommendations of the 'Union Internationale de Chimie' as drawn up by Karrer (1948). A full investigation into this pigment must await the availability of larger amounts of ripe *Lonicera* berries at a time when it is feasible to examine them. The discovery of a more potent source of this pigment would also be useful in this connexion, for, as will be seen later (Table 6), η-carotene is only a minor component (1·3 %) of the total carotenoids of *Lonicera*.

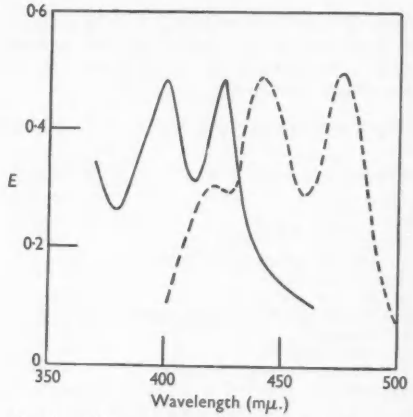

Fig. 1. Absorption spectrum of new carotene (η-carotene) obtained from *Lonicera* berries compared with that of ε-carotene from the alga *Navicula torquata*. ——, η-carotene in light petroleum (b.p. 40–60°); – – – –, ε-carotene in ethanol (from Strain & Manning, 1943). Note. (i) The change in the position of absorption maxima of carotenes in light petroleum and ethanol is only slight (2–4 mμ.); (ii) the *E* values are arranged so that $E_{max.}$ is the same for both pigments.

Fraction 4. This fraction is epiphasic to 90 and 95 % (v/v) aqueous methanol, has an absorption spectrum almost identical with that of β-carotene, and is adsorbed on alumina to very much the same degree as is free vitamin A. It is probable that this is the unidentified pigment found in human blood serum and milk by Kon & Mawson (1950). Their pigment showed absorption bands at 450 and 476 mμ., was adsorbed more strongly than lycopene and could not be separated chromatographically from vitamin A.

Fraction 5. This fraction occurred only in minute traces. It exhibited an absorption spectrum similar to that of β-carotene; insufficient amounts were available for further study.

B. *The xanthophylls*

Fraction 6*A*. This fraction was identified as cryptoxanthin; it had the same absorption spectrum (Fig. 2) and the same chromatographic properties as authentic cryptoxanthin. Furthermore, in the partition test, it was not extracted from light petroleum by shaking with 90 % (v/v) aqueous methanol; when, however, 95 % (v/v) aqueous

methanol was used the pigment was equally distributed in the two phases; this is a characteristic property of cryptoxanthin.

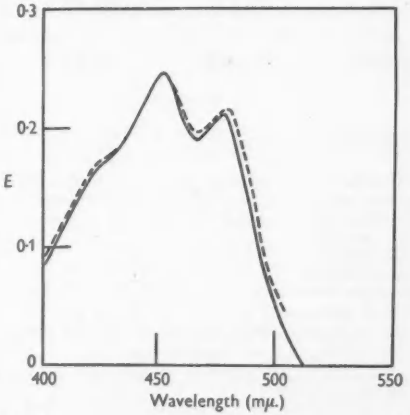

Fig. 2. A comparison of the absorption spectrum of the pigment (zone 6*A*, Table 3) obtained from *Lonicera* berries with that of authentic cryptoxanthin obtained from maize. The *E* values are so arranged that $E_{max.}$ is the same for both pigments. – – – –, *Lonicera* pigment; ——, cryptoxanthin. Solvent, light petroleum.

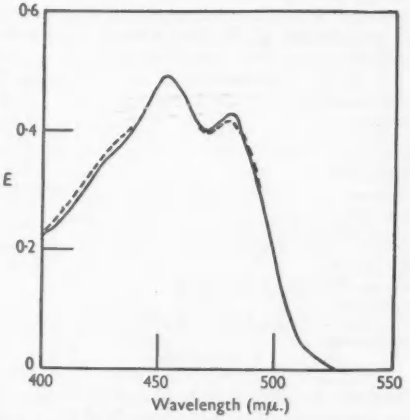

Fig. 3. A comparison of the absorption spectrum of the pigment (zone 6*C*, Table 3) obtained from *Lonicera* berries with that of authentic zeaxanthin obtained from maize. The *E* values are so arranged that $E_{max.}$ is the same for both pigments. – – – –, *Lonicera* pigment; ——, zeaxanthin. Solvent, ethanol.

Fraction 6*C*. A fraction which was completely hypophasic in the partition tests was identified as zeaxanthin by comparison in the usual ways with a specimen of zeaxanthin obtained from maize. A comparison of the spectrum of the berry pigment with that of authentic zeaxanthin is recorded in Fig. 3.

Fraction 6*DB*. This fraction appears to be auroxanthin. No authentic auroxanthin was available for comparison, but the very characteristic properties of this pigment leave little, if any, doubt as to its identification. The position and shape of its absorption spectrum is shared by only two other carotenoids, ζ-carotene and aurochrome (and to a lesser degree by η-carotene). A consideration of other properties of these four pigments (Table 5) indicates that the present pigment can only be auroxanthin. A comparison of the spectra of pigment 6*DB* and that of auroxanthin is recorded in Fig. 4.

Fractions 6*B*, 6*DA*. The spectra are recorded in Fig. 4, but could not be identified with any known xanthophylls. The compounds are completely hypophasic. The poor persistence of their spectra suggests that they might be neoxanthophylls (compare the spectral persistence of the neofucoxanthins (Strain, Manning & Hardin, 1943)). Because of the well-known lability of xanthophylls, much further work is necessary before the possibility that these pigments are not merely oxidative artifacts can be completely disproved.

Natural occurrence of the xanthophylls

Partition experiments before saponification showed that the pigments in the crude extract of the berries were almost completely epiphasic using both 90 and 95% (v/v) aqueous methanol, thus indicating that the xanthophylls occurred naturally almost entirely as esters.

The quantitative distribution of the component pigments

The relative amounts of the pigments present in ripe *Lonicera* berries are recorded in Table 6. It will be clearly seen that cryptoxanthin is the predominant pigment.

DISCUSSION

A great deal of the classical work on the isolation of carotenoids for determination of structure has been carried out on fruit of various species (see Karrer & Jucker, 1949, for a complete survey) and, quite naturally, with the emphasis being on isolation of large amounts, minor components were often ignored. Now that, as a result of these investigations, the properties of so many carotenoids are accurately known, it is possible, by utilizing improved chromatographic and spectrographic techniques, to separate and identify carotenoids without the necessity of isolating them in crystalline form. In this way it has recently been shown that tomatoes

Table 5. *A comparison of the properties of pigment 6DB (Table 3) with those of auroxanthin, aurochrome, ζ-carotene and η-carotene*

Property	Pigment 6*DB*	Auroxanthin	Aurochrome	ζ-Carotene	η-Carotene
Absorption maxima in light petroleum (mμ.)	400, 425	400, 425	428*	400, 426	399, 425
Partition between light petroleum and 90% (v/v) aqueous methanol	Hypophasic	Hypophasic	Epiphasic	Epiphasic	Epiphasic
Colour with conc. HCl	Stable blue	Stable blue	Stable blue	None	None
Position on chromatogram	Strongly adsorbed above zeaxanthin	Strongly adsorbed above zeaxanthin	Can be developed on $Ca(OH)_2$ with light petroleum; hydroxycarotenoids are not developed with this solvent	Adsorbed above β-carotene but below lycopene	Adsorbed below α-carotene
References	Present work	Karrer & Rutschmann (1942)	Karrer & Jucker (1945, 1949)	Goodwin (1952*a*) and present work	Present work

* The lower wave band in light petroleum is not recorded by Karrer & Jucker probably for technical reasons. Its existence is, however, obvious from a reference to the curve for aurochrome in CS_2.

contain in addition to lycopene (the major component) very small amounts of a series of polyenes, each differing from the next in the series by four hydrogen atoms (Porter & Lincoln, 1950). From this investigation it has been postulated that these pigments represent successive intermediate steps in the synthesis of the fully unsaturated carotenoids (lycopene, β-carotene, etc.). The present work indicates that a very similar series of pigments are present in *Lonicera* berries and this points to a pathway of synthesis similar to that occurring in tomatoes. As such a series of pigments has never been demonstrated in leaves, this strongly suggests that the route of carotenoid biogenesis in fruit is fundamentally different from that in green leaves.

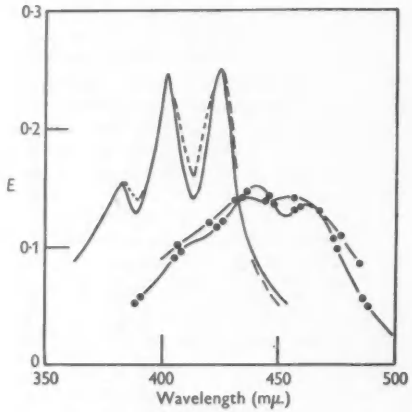

Fig. 4. The absorption spectra of the pigment 6*DB* (Table 3), authentic auroxanthin, and unidentified xanthophylls 6*B* and 6*DA* (Table 3). ——, authentic auroxanthin in ethanol (redrawn from Karrer & Rutschmann, 1942); - - - -, pigment 6*DB* in ethanol; —·—·—, pigment 6*B* in benzene; ···—···—, pigment 6*DA* in ethanol. The *E* values for pigment 6*DB* and auroxanthin are so arranged that $E_{max.}$ is the same for both pigments. The *E* values for the other two pigments are those observed in solutions of unknown strength. They bear no relationship to each other or to the *E* values of 6*DB* and auroxanthin.

Table 6. *The quantitative distribution of the polyene components present in* Lonicera *berries, measured as percentages of the total amount of pigments present*

Carotenoid	Amount
Phytofluene-like (Fraction 1*A*, Table 2)	0·45
η-Carotene	1·9
Phytofluene	1·3
β-Carotene	8·95
ζ-Carotene	0·6
γ-Carotene	0·6
Lycopene	2·5
Human milk pigment (Fraction 4, Table 1)	2·9
Unknown (Fraction 5, Table 1)	Trace
Cryptoxanthin	65·2
Unknown (Fraction 6*B*, Table 3)	1·9
Zeaxanthin	3·5
Unknown (Fraction 6*DA*, Table 3)	5·2*
Auroxanthin	5·0

* By difference.

Recent work has revealed the presence of a very similar series of polyenes in the fungus *Phycomyces blakesleeanus* (Goodwin, 1952*a*), and earlier work suggests that most of the components of the series also exists in other carotenogenic fungi; the synthetic route in fruit and fungi may thus be very similar. As yet, no such polyene series has been demonstrated in algae, flower petals or bacteria.

Phytofluene, a member of this series, has, however, been observed in a number of flower species (Zechmeister & Sandoval, 1945), and in one bacterium, *Mycobacterium phlei* (Goodwin, 1952*a*). In the photosynthetic bacterium, *Rhodospirillum rubrum*, however, preliminary experiments suggest that phytofluene is not present (Goodwin & Osman, 1951).

The nature of η-carotene

The position of the absorption spectrum of η-carotene, which is very similar to that of ζ-carotene, suggests that, like ζ-carotene, it contains seven conjugated double bonds. As it is less strongly adsorbed on a column than is ζ-carotene, and as its position on the column is the same relative to β-carotene as that of ζ-carotene is to lycopene, it is possible that η-carotene bears the same structural relationship to β-carotene as ζ-carotene does to lycopene. Thus, it might well be octahydro-β-carotene, with the double bonds symmetrically placed about the centre of the molecule. If either of the double bonds in the β-ionone residues were concerned in the chromophoric system, then one would expect the position of the absorption bands of ζ-carotene to be different from those of η-carotene, in the same way as those of lycopene are different from those of β-carotene.

The small amount of material with a spectrum similar to that of phytofluene, which has been observed to be adsorbed below η-carotene may be, on similar reasoning, dodecahydro-β-carotene, i.e. the β-carotene derivative corresponding to phytofluene, which is probably dodecahydrolycopene (Porter & Lincoln, 1950).

It will be seen from Table 6 that *Lonicera* berries fall into one of the two main categories of carotenoid-containing fruit: those having cryptoxanthin as their major component. The other group tends to accumulate large amounts of lycopene.

The occurrence in the berries of a pigment (fraction 4, Table 1) which appears to be the 'unidentified pigment' observed in human blood serum and milk by Kon & Mawson (1950) is important because this is the first time it has been reported in plant tissue. Willstaedt & With (1938), who observed a similar pigment in blood serum, considered it to be an 'oxidation product'. This possibility remains, but now the further possibility exists that it occurs in human blood serum and milk as a result of its ingestion in the food.

Two final points of interest may be mentioned: (1) neither α-carotene nor any of its derivatives occurs in the berries; and (2) this is the first time that auroxanthin has been observed in berries, although it is widespread in flower petals (Karrer & Jucker, 1949).

SUMMARY

1. The following known carotenoids have been found in the ripe berries of the climbing honeysuckle (*Lonicera japonica*): phytofluene, β-carotene, ζ-carotene, γ-carotene, lycopene, cryptoxanthin, zeaxanthin and auroxanthin; an unidentified pigment present in human blood and milk (Kon & Mawson, 1950) also appears to be present.

2. Some spectral and adsorption properties of a new carotene (η-carotene) occurring in the berries are described. Small amounts of a polyene very similar to, but distinct from, phytofluene were also observed.

3. Three pigments occurring in small amounts were not identified; their spectra are recorded.

4. The relative amounts of the pigments present have been determined; cryptoxanthin is the major component. The xanthophylls exist almost exclusively as esters.

5. This work provides additional evidence to support the suggestion that the route of carotenogenesis in fruit is different from that in green leaves.

Thanks are due to Prof. R. A. Morton, F.R.S., for his interest in this work.

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The Application of Counter-Current Distribution to the Separation of Phospholipins

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Counter-current distribution between two immiscible solvent phases, as first developed by Craig (1944), offers advantages over both direct and partition chromatography for analytical and preparative work in the field of phospholipins (Lovern, 1949). The only recorded use of the method for such purposes appears to be in a series of papers from one laboratory and deals with vegetable phosphatides (Scholfield, Dutton, Tanner & Cowan, 1948; Scholfield, McGuire & Dutton, 1950; McGuire & Earle, 1951). These workers obtained varying degrees of separation of constituents by distribution between hexane and aqueous methanol or ethanol in a metal apparatus of essentially the original Craig pattern. Various developments in design of apparatus (Lochte & Meyer, 1950; Craig, Hausmann, Ahrens & Harfenist, 1951; Lathe & Ruthven, 1951) have considerably extended the scope of the method, and recently the similar 'cascade distribution' (Kies & Davis, 1951) offers an alternative technique.

The author has studied the applicability of the counter-current technique to the separation of the constituents of the crude glycerophosphatide fraction of the lipids of ox brain, using light petroleum (b.p. 40–60°) and aqueous ethanol as solvents. Preliminary tests, both in a metal apparatus of the original Craig pattern and in a series of separating funnels, suggested that phospholipins are likely to give trouble with apparatus in which all equilibrations and transfers are performed by one operation. They appear to have a considerable effect on the solubility of one solvent in the other. Thus, although the two phases are saturated with each other at the start of the experiment, the relative volumes of the two phases alter appreciably as fractionation proceeds along a series of vessels. This could lead to trouble with mixed phase transfers in an automatic apparatus. Moreover, at certain stages of the separation—usually rather late stages—it occasionally happens that the contents of one vessel will form a very stable emulsion whereas all the rest separate rapidly. This may occur only once or twice in the course of hundreds of transfers, and it can easily be dealt with in a procedure based on individual transfers, but would pass unnoticed in an automatic apparatus. For these reasons, in spite of the great expenditure of time required, the use of a series of conical flasks has been adopted. They permit the use of any desired volumes and are thus ideal for preparative work. Separating funnels are more easily manipulated, but involve the danger of contamination with stopcock grease. Beroza (1951) prefers bottles to funnels for the same reason.

EXPERIMENTAL

The main experiment was carried out with the total ether-soluble fraction of the crude phospholipins from ox brain. It would, therefore, contain all those lipids based on glycerophosphoric acid (except part of the fully saturated ones, e.g. dipalmityl lecithin), but should be almost free from sphingolipins. Subsidiary trials, for comparative purposes, were made with smaller amounts (about 7 g. each) of crude preparations of (*a*) phosphatidyl choline, (*b*) phosphatidyl ethanolamine and (*c*) phosphatidyl serine. Preparation (*a*) contained much (*b*), (*b*) contained minor amounts of (*a*), and (*c*) contained a little (*b*). Preparations (*b*) and (*c*) were made by the method of Folch (1949) from the 'kephalin' of mixed ox brain and spinal cord lipids. All products contained plasmalogen, a constituent which seems to have been ignored by Folch (cf. also Klenk & Böhm, 1951). They were free from acetone-soluble lipids but had not been purified by dialysis.

Ox-brain glycerophosphatide (120 g.) was dissolved in 300 ml. of light petroleum saturated with 85% (v/v) ethanol in the first of a series of twenty conical flasks, the other flasks likewise containing 300 ml. of light petroleum saturated with 85% ethanol. Batches of 300 ml. of 85% ethanol saturated with light petroleum were passed in succession through the train of flasks, with thorough shaking at each stage. Transfer from flask to flask was effected by suction. The successive ethanol extracts were removed as a series of fractions from the end of the series, the process corresponding roughly to elution chromatography. The operation was continued until the weight of lipid in each fraction was very small and only a small proportion of the total lipid remained in the petroleum phase. This gave 62 eluted fractions accounting in all for 93·3% of the total material. The contents of each petroleum fraction were separately recovered, giving a further 20 fractions containing the remaining 6·7% of the original lipid.

The supplementary trials with crude phosphatidyl serine and crude phosphatidyl ethanolamine were run in the same manner, using 100 ml. batches of each solvent phase, in a series of twenty conical flasks, except that only 20 ethanol fractions were run through the train in the former case and, inadvertently, 19 instead of 20 fractions in the latter. The eluted material amounted to 83·3% of the total for phosphatidyl serine and 69% for phosphatidyl ethanol-

amine. The crude phosphatidyl choline was treated differently. It was partitioned between batches of 100 ml. each of light petroleum and 90% (v/v) ethanol in a series of sixteen flasks, but no fractions were run off at the end of the series. Instead, the ethanol phases were allowed to remain successively one flask nearer the first in the series, until all flasks contained both phases. Then the entire contents of each flask were recovered as a single fraction. This corresponds to development of a chromatogram until the solute just reaches the bottom of the column, followed by cutting of the column into sections for extraction.

Grünberg (1938) which, although less reliable than that of Ehrlich, Taylor & Waelsch (1948), involves a simpler technique adequate for observing the progress of separation among a large number of fractions. $CHCl_3$ was used instead of amyl alcohol as the extractant, since it gives much lower blank values. Comparison was against a standard of pure palmitaldehyde, used as such and not as its glyceryl acetal. Palmitaldehyde was kindly supplied by Mr G. I. Gregory, of the Department of Organic Chemistry, University of Bristol. Phosphorus was determined on certain fractions, using Allen's (1940) method.

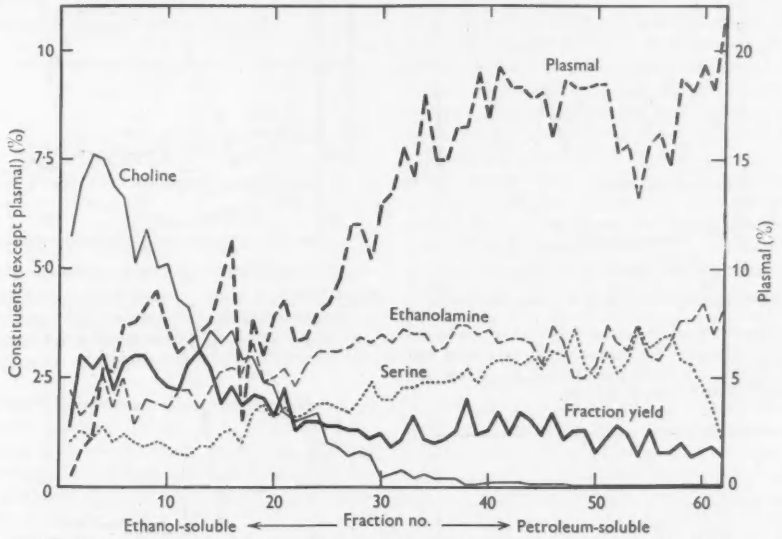

Fig. 1. Ethanol phase in the distribution of crude ox-brain glycerophosphatides between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

All fractions were examined for choline, ethanolamine, serine and plasmals (higher fatty aldehydes). Inositol was not determined. Paper chromatography (Chargaff, Levine & Green, 1948; Brante, 1948) was used for qualitative examination of the bases present. Choline was determined after refluxing for 2 hr. with ethanolic 0·5 N-KOH, followed by addition of water, acidification with HCl, removal of fatty acids with ether and evaporation of the aqueous solution to dryness on the steam can under vacuum, to remove excess HCl. The residue was made up to known volume with water (saturated with $CHCl_3$ to prevent microbial destruction if any appreciable delay should occur) and the choline estimated by Glick's (1944) method, combined with the use of Beattie's (1936) aqueous reineckate solution and Winzler & Meserve's (1945) ultraviolet absorption technique. Ethanolamine and serine were determined after refluxing for 6 hr. with 6 N-HCl, removal of fatty acids with ether, evaporation to dryness and solution in water to known volume as for choline. The ethanolamine and serine content was then determined by a combination of the methods of Artom (1945) and Burmaster (1946), permutit being replaced by the synthetic resin Zeo-Karb 215. Plasmals were determined by the method of Feulgen &

RESULTS AND DISCUSSION

Fig. 1 shows the results obtained for the 62 fractions of the ethanol phase of the ox-brain glycerophosphatides, for yield (percentage of total ethanol-phase lipid) and content of the three nitrogenous components and of plasmals, all expressed as percentages. Fig. 2 shows similar results for the 20 fractions of the petroleum phase, except that serine and ethanolamine have been given as total amino base. This was predominantly ethanolamine. The results obtained on the crude phosphatidyl choline are shown in Fig. 3, those for the ethanol phase of crude phosphatidyl ethanolamine in Fig. 4 and for the petroleum phase of the same fractionation in Fig. 5. Fig. 6 shows the data obtained on the ethanol phase of the crude phosphatidyl serine, most of the petroleum phase fractions being too small for analysis. In Figs. 1 and 4 the plasmal scale is half that for the other components, but in the other diagrams it has proved convenient to use the

same scale for all components. To facilitate comparison of the various Figures, fraction numbering in all cases has been arranged so that number 1 represents the fraction most preferentially soluble in the ethanol phase.

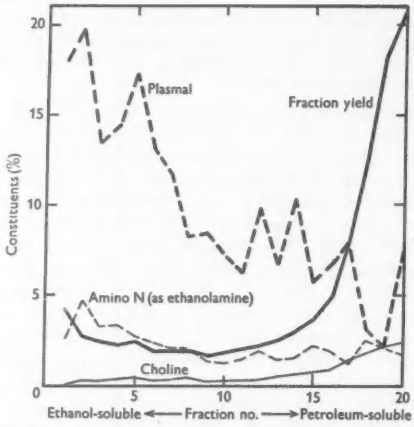

Fig. 2. Petroleum phase in the distribution of crude ox-brain glycerophosphatides between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

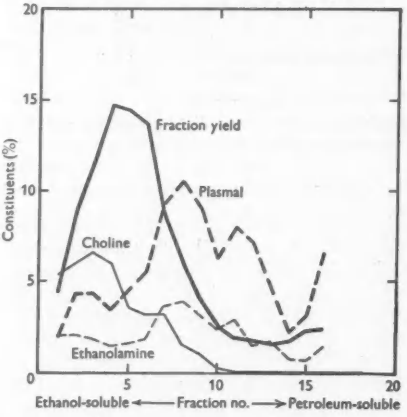

Fig. 3. Combined phases in the distribution of crude phosphatidyl choline between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

Considering first the main experiment, the fraction-yield curve in Fig. 1 indicates a whole series of overlapping fractions and various changes in appearance of the fractions also suggest a complex distribution pattern. Fractions 1 and 2 were deep orange in colour and only semi-solid at room temperature. Fraction 3 was a little paler and more solid. Fractions 4–7 were yellow and quite firm solids, fractions 8–12 were cream-coloured firm solids, fractions 13 and 14 were yellow soft semi-

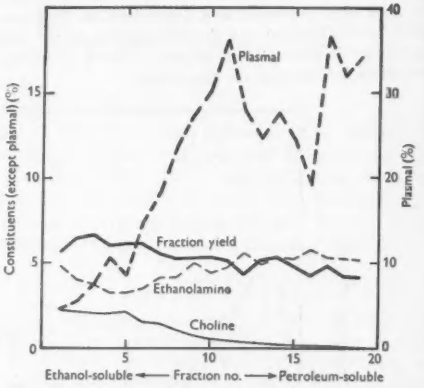

Fig. 4. Ethanol phase in the distribution of crude phosphatidyl ethanolamine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

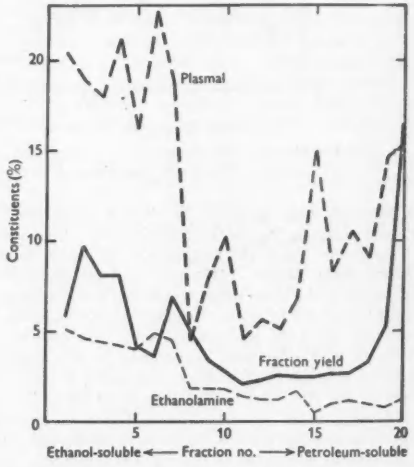

Fig. 5. Petroleum phase in the distribution of crude phosphatidyl ethanolamine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

solids, fraction 15 was again cream coloured and somewhat firmer, fractions 16–57 were cream-coloured firm solids and fractions 58–62 were yellowish brown firm solids.

As might be expected from the known solubility properties of the phospholipins, phosphatidyl choline is removed by the ethanol preferentially to most of the other constituents, but the curve indicates the presence of derivatives of considerably different partition coefficients, varying presumably in fatty acid components. Some of these, e.g. the small proportion of the total phosphatidyl choline represented by fractions 30–37, are markedly different from the main fraction represented by fractions 1–7. Phosphatidyl choline is not completely extracted until fraction 49.

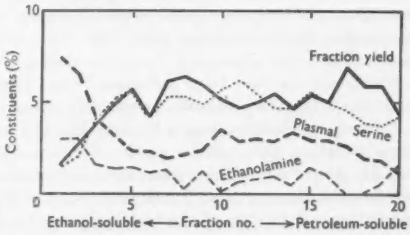

Fig. 6. Ethanol phase in the distribution of crude phosphatidyl serine between light petroleum and aqueous ethanol. All curves pass through experimentally determined values for every fraction.

Figs. 3 and 4 likewise show the early extraction of phosphatidyl choline by the ethanol. The lipid preparations in question had, of course, already been separated on the basis of relative solubility in ethanol, and as the starting material (mixed brain and spinal cord as against brain only) was also different, the curves would not be expected to duplicate very closely those in Fig. 1. It may be significant that the long tail-off in the extraction of phosphatidyl choline appears in Fig. 4 for the less ethanol-soluble preparation, but is not seen in Fig. 3. On the other hand, Fig. 3 shows evidence of at least two phosphatidyl cholines, one represented by fractions 1–4, and one by fractions 5–7. These may well correspond to fractions 1–12 and 13–18 respectively in Fig. 1.

It might be expected that the curve for ethanolamine would be particularly complex, since this base is present in two lipid classes, phosphatidyl ethanolamine and plasmalogen. In Fig. 1, from fraction 1 to about fraction 22, the ethanolamine curve shows violent fluctuations, with indications of some large main fractions, e.g. fractions 13–22 inclusive. In these first 22 fractions, apart from the earliest ones and, apparently, fraction 17, the ethanolamine is derived from both the above types of phospholipin. A plasmal content of 7·5%, about the mean level of fractions 8–22, would account for an ethanolamine content of about 1·8%, whereas the total ethanolamine content of these fractions is of the order of 2·5%. The plasmal curve covering these same 22 fractions shows evidence of more than one plasmalogen, presumably involving a different aldehyde. There is considerable discrepancy in the literature as to the range of aldehydes present in brain plasmalogen (Thannhauser, Boncoddo & Schmidt, 1951; Leupold, 1950). At the time the present work was completed it had not been reported that plasmalogens may have a base other than ethanolamine, but some of the results, discussed later, suggest that this must be the case. A very recent paper by Klenk & Böhm (1951) provides evidence for a plasmalogen containing serine instead of ethanolamine. In spite of the relatively inaccurate technique, there are clearly three distinct plasmalogen fractions represented roughly by fractions 1–11, 12–17 and 18–22 respectively.

From fractions 23 to 44 inclusive, the ethanolamine curve shows only small fluctuations, some within the limits of experimental error. Over this same range of fractions, the plasmal curve climbs steadily to a roughly constant level of 17%, which is then maintained from about fractions 42 to 51. Some of the peaks and depressions in the plasmal curve over the fraction range 23–44 can be correlated with similar features in the ethanolamine curve, e.g. the drop in both curves for fractions 35 and 36, but many of the smaller irregularities in the plasmal curve may be due to experimental error. A plasmal level of 17% requires about 4% of ethanolamine, fully as much as the total present. Although the estimation of natural plasmalogen against free palmitaldehyde is not likely to give an accurate figure for the plasmal content of the fractions, its approximate correctness was confirmed by estimation of the acetone-soluble lipid set free when an ether solution of the fraction was emulsified with a solution of mercuric chloride, which rapidly breaks the acetal linkage in plasmalogen (Feulgen, Imhäuser & Behrens, 1929).

From fraction 45 onwards the ethanolamine curve again shows considerable fluctuations with evidence of at least three main components: fractions 45–48, 49–57 and 58–62 respectively. Some fractions show trends in the ethanolamine curve in this range corresponding to similar trends in the plasmal curve, e.g. the very low value for fraction 57, but most of them are entirely unconnected, e.g. the ethanolamine peak at fraction 54 is associated with a pronounced minimum on the plasmal curve. There is, rather, evidence in fractions 45 to at least 57 of the occurrence of phosphatidyl ethanolamines different from those of fractions 1–22, a point referred to again in considering the serine curve. The plasmal curve shows clear evidence of more than one fraction over this later range, the minimum between fractions 51 and 58 being outstanding.

The results on crude phosphatidyl choline (Fig. 3) show a much closer parallelism between the distribution of plasmalogen and ethanolamine. They agree with Fig. 1 in suggesting more than one plasmalogen (fractions 1–4, 5–14, 15 and 16). Again it is in the earlier fractions that the highest ratios of phosphatidyl ethanolamine to plasmalogen are found. At the peak of the plasmal curve there is still a considerable excess of ethanolamine (4 %) over that required by the plasmal (about 2·4 %), but at fraction 16 the ratio of plasmal to ethanolamine is just right for plasmalogen.

In Fig. 4, representing a relatively less ethanol-soluble lipid than Fig. 3, there is evidence of two plasmalogens, the plasmal curve corresponding fairly well to the later part of that in Fig. 1 (from fraction 17 onwards). The ethanolamine curve does not run parallel with it and, as in the other cases, ethanolamine differs from choline in not showing any tendency to elimination as fractionation proceeds. Fig. 4 emphasizes that lipids rich in ethanolamine tend to be rich in plasmalogen, but it also provides direct evidence that not all this plasmal can be present in ethanolamine-containing plasmalogen. Some of the fractions contain about 36 % of plasmal, requiring about 8·6 % of ethanolamine, whereas only 5 % is present. At fraction 16 the ratio of the two substances is about right for the accepted plasmalogen structure. In the earlier fractions there is a great excess of ethanolamine, obviously present as phosphatidyl ethanolamine.

In the partition of the crude phosphatidyl ethanolamine the process was not carried near to completion, and Fig. 4 should be compared with the early part of Fig. 5. For fractions 1–7 (Fig. 5) the plasmal content of about 20 % requires about 4·8 % of ethanolamine, near to the 4·5 % which is present. The later fractions in Fig. 5, however, tell a different story. They contain 10–15 % of plasmal, requiring about 3 % of ethanolamine, whereas only about 1 % is found.

The crude phosphatidyl serine, Fig. 6, contains very little plasmalogen, and where ethanolamine is present it is more than adequate to account for the plasmal. But in one or two fractions no ethanolamine at all could be detected, yet plasmal in about the same amount as in the neighbouring fractions was still present.

The serine curve in Figs. 1 and 6 resembles the ethanolamine curve in being extended over the full range of fractions. In Fig. 1 it shows only minor fluctuations from fractions 1 to 17, though with a definite minimum in fractions 11 and 12, after which there is a sudden increase followed by a fairly general rise with minor fluctuations to fraction 44. From here until fraction 57 the serine curve exhibits a series of abrupt rises and falls rivalling those of ethanolamine over the same fraction range, the two curves being sometimes in phase and sometimes out of phase. In general, the similar behaviour of phosphatidyl ethanolamine and phosphatidyl serine is not surprising, in view of the extreme difficulty in separating these lipids by the classical methods. The curves in Fig. 1 suggest that they have a similar assortment of fatty acids and that both contain representatives, appearing in fractions 45 onwards, considerably different in fatty acid composition from those appearing in the earlier fractions. Klenk & Böhm (1951) emphasize the complex range of fatty acids present in both phospatidyl ethanolamine and phosphatidyl serine, but report great differences in this respect between the two lipids. However, they mention that intermediate fractions, discarded in the preparation of purified fractions, may have influenced this. It was noted that fractions 45–62 (especially fractions 60–62) were much more slowly broken down by acid hydrolysis than were all the earlier fractions. The generally slower rise of the serine curve until it meets the ethanolamine curve at fraction 45 is in line with the relative solubilities of the two lipids in ethanol (Folch, 1948).

The serine curve in Fig. 6 exhibits a steep initial rise instead of the long slow rise in Fig. 1. There are several peaks and depressions, the agreement between Figs. 1 and 6 showing that there must be a number of phosphatidyl serines, differing in their fatty acid components.

In all cases the combined derivatives of choline, ethanolamine and serine can account for only a portion of the total fraction, e.g. ranging for Fig. 1 from 48 to 89 %, with a value of about 60 % for the majority of the fractions. Phosphorus determinations, made on every fifth fraction in Fig. 1, ranged from 2·0 to 3·7 %, with most in the range 2·5–3·5 %, thus corresponding quite well with the values obtained from the nitrogenous derivatives. Part of the balance in some fractions may consist of inositol lipids, but a much greater factor seems to be the presence of lipids of the wax ester type. All the fractions in Fig. 1 yield appreciable amounts (ranging from 3 to 15 %, with the lowest values in the early fractions) of unsaponifiable matter, by ether extraction of alkaline hydrolysates. According to Feulgen *et al.* (1929) the acetal linkage of plasmalogen is completely resistant to 5 hr. boiling with 5 % aqueous sodium hydroxide, and ether extraction of the solution should leave the aldehyde in the aqueous layer as the sodium salt of plasmalogenic acid. In the author's laboratory, where ethanolic 0·5 N-potassium hydroxide is routinely used for alkaline hydrolysis, it has been found that small, variable amounts of plasmal are set free in 2 hr. refluxing. Accordingly, unsaponifiable matter has been determined after removal of plasmal with mercuric chloride. The unsaponifiable matter from

all fractions had a similar appearance, being a soft paste at room temperature and a mobile liquid when warm. It had an iodine value ranging from 70 to over 100 in the various fractions. The total product was submitted to fractional distillation at 0·1 mm. pressure, but only a few drops distilled over the range 120–180°, when distillation was stopped. Distillate and residue were recombined and acetylated. The product had a saponification equivalent of 411·3, corresponding approximately to the acetate of a C_{25} monohydric alcohol. There would almost certainly be a mixture of homologues present, probably those of an even number of carbon atoms. Such alcohols, presumably esterified with fatty acids, do not seem to have been reported previously in the crude phospholipin fraction of ox brain.

Turning to Fig. 2, and comparing it with Fig. 5, it can be seen that the greater part of the material recovered from the petroleum phase has remained virtually unmoved by the ethanol. Following the initial fractions containing material closely resembling the final fractions of the ethanol phase, especially marked in the incomplete procedure represented in Fig. 5, there is a long run of very small fractions and then an abrupt rise.

The nature of the last few fractions is obscure. In Fig. 2 appreciable amounts of choline are shown, which cannot be present as phosphatidyl choline. The obvious suggestion would be small amounts of sphingomyelin but these fractions do not have the solubility properties of sphingomyelin. They are firm solids readily soluble in cold ether or light petroleum. They are quite insoluble in ethanol. They also contain appreciable amounts of plasmal and ethanolamine. It should be noted that they account for only a very small proportion (less than 0·5 %) of the total for the crude glycerophosphatides of ox brain, but considerably more—about 8 %—of the crude phosphatidyl ethanolamine of the mixed brain and spinal cord. The very small fractions covering the long flat minimum of the petroleum phase-yield curve are different again, being soft gummy products which become liquid on warming.

It seems clear that such a complex mixture as the total glycerophosphatides of ox brain requires a far longer fractionation chain than 20 units to effect sharp separation of major constituents, and that even under the best conditions there would be poor separation of some of them, e.g. of serine-containing from ethanolamine-containing lipids, unless other solvent pairs should give better results. However, in Fig. 1, fractions 1–20 contain most of the phosphatidyl choline with relatively small proportions of lipids containing serine and ethanolamine. Fractions 30–45 should give a fraction consisting mainly of plasmalogen and phosphatidyl serine, and fractions 58–62 should contain an even purer plasmalogen. When it is remembered how markedly phospholipins influence the solubility properties of other phospholipins it is not surprising that sharp separation is not achieved by the relatively simple fractionation procedure used in these experiments.

SUMMARY

1. The crude glycerophosphatides of ox brain, and crude preparations of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine, have been partitioned between aqueous ethanol and light petroleum, employing the technique of counter-current distribution.

2. The results suggest a useful degree of separation of phosphatidyl choline from similar lipids containing ethanolamine and serine and of plasmalogen from lipids other than phosphatidyl serine. Separation of phosphatidyl ethanolamine from phosphatidyl serine is poor.

3. There is evidence that the plasmalogens are a complex group which may include derivatives with a base other than ethanolamine, as well as a range of aldehydes. The glycerophosphatides containing choline, ethanolamine and serine all show evidence of multiplicity of fatty acid composition.

4. The crude phospholipins of ox brain contain substances not so far classifiable, including choline-containing lipids different from lecithin and sphingomyelin, appreciable amounts of what appear to be wax esters, and gummy lipids of unknown composition.

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Estimation of Nucleic Acids in Tissue from the Nervous System

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The starting point of this investigation was the report of Davidson & Waymouth (1944) that white matter of the brain of the sheep contained a higher concentration of protein-bound phosphorus, i.e. phosphorus remaining after removal of lipid phosphorus and acid-soluble phosphorus, than grey matter. If most of this protein-bound phosphorus were present as nucleic acid, there remained the possibility that nucleic acid might be an important constituent of the myelin sheath of a mammalian nerve fibre. Since no nucleic acid can be detected in the myelin sheath by the usual histochemical means, it seemed worth while to investigate the matter further. Attempts were made to estimate the concentration of deoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) in different parts of the nervous system by two methods at present widely used, that of Schmidt & Thannhauser (1945) and that of Schneider (1945). Although recovery experiments showed that added DNA, PNA, or mixtures of the two, could be recovered quantitatively from brain extracts by either method, it soon became apparent that the two methods gave widely diverging results when they were applied to white matter and grey matter of brain and to peripheral nerve.

It was decided, therefore to investigate the validity of these methods for tissue from the nervous system. The conflicting results are due, in part, to the presence of considerable amounts of a phosphorus-containing compound in brain and spinal cord, particularly in the myelin-rich white matter. The phosphorus of this compound is not removed by ice-cold 10% (w/v) trichloroacetic acid (TCA) nor is it removed by the usual ethanol-ether treatment for the extraction of lipids. This substance is probably the same as the inositol-containing trypsin-resistant lipid-protein complex isolated from white matter by Folch & Le Baron (1951). Dr J. Folch, McLean Hospital, Waverley, Mass., in experiments as yet unpublished, has obtained results similar to those reported here.

In addition, it is shown that TCA extracts of brain and nerve tissue, prepared as described by Schneider (1945), contain chromogenic material that interferes with the colour reactions of Dische (1930) for DNA and Mejbaum (1939) for PNA. Folch (1951) suggests that this interference may be caused by traces of the complex lipid substance, strandin, isolated from brain by Folch, Arsove & Meath (1951). A preliminary account of these experiments has already appeared (Rossiter, Logan & Mannell, 1951).

METHODS

Standards

The same DNA and PNA standards were employed throughout this work. Results obtained for each of the methods are comparable inasmuch as they are all given in terms of the P content of these standards. The DNA standard was a preparation of calf sodium thymonucleate prepared by the method of Mirsky & Pollister (1942), and kindly provided by Dr G. C. Butler, Department of Biochemistry, University of Toronto. The PNA standard was a preparation of yeast sodium ribonucleate selected from a number of commercial preparations examined. Table 1 shows that the absorption

Table 1. *Absorption characteristics and phosphorus content of standard preparations of DNA and PNA*

	$\lambda_{max.}$ (mμ.)	ϵ (P) at $\lambda_{max.}$	$\lambda_{max.}$ (mμ.)	ϵ (P) at $\lambda_{min.}$	P (%)
DNA	259	7300	230	2900	8·3
PNA	258	9300	228	3500	8·5

characteristics and P content of the standards are in good agreement with the published figures for DNA and PNA (Stimson & Reuter, 1945; Di Carlo & Schultz, 1948; Blout & Fields, 1949; Chargaff *et al.* 1949, 1950).

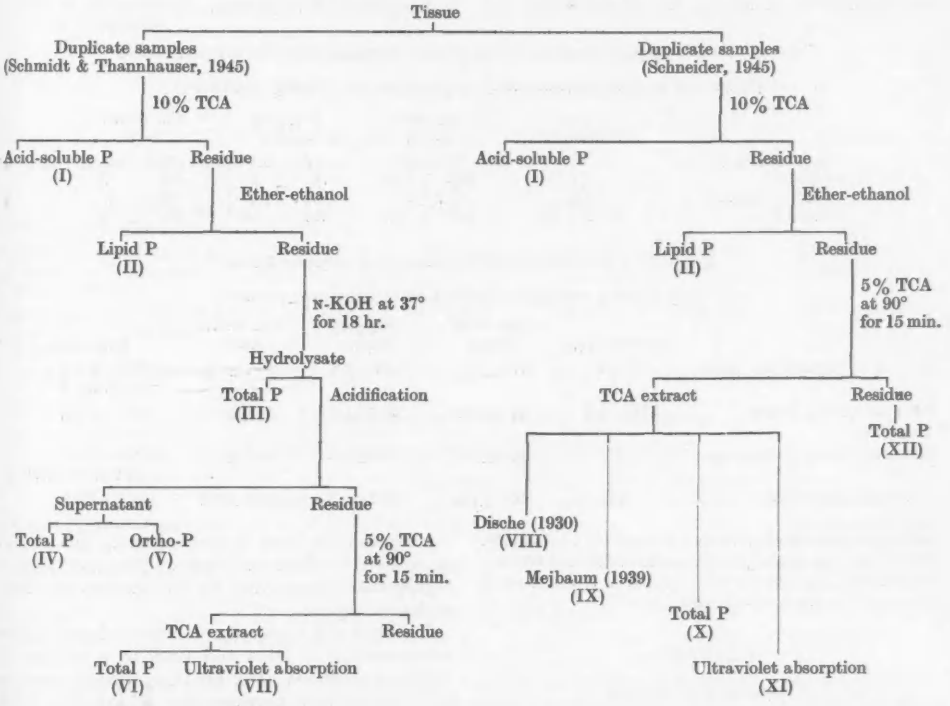
The symbol ϵ (P) is used for the atomic extinction coefficient with respect to P as defined by Chargaff & Zamenhof (1948), i.e. $\epsilon(P) = \frac{30{\cdot}98\,E}{cl}$, where E is the extinction ($\log I_0/I$), c is the concentration of P in g./l. and l is the thickness of the absorbing layer in cm. The value of ϵ (P) at $\lambda_{max.}$ is a little greater than that given by Chargaff *et al.* (1949). The value of ϵ (P) probably depends upon the degree of polymerization of the sample (Chargaff *et al.* 1950). For samples of thymus DNA, presumably less highly polymerized, Stimson & Reuter (1945) and Blout & Fields (1949) reported much higher values of ϵ (P).

matter was chiefly corpus callosum. The sciatic nerve of the cats was rapidly cleaned of adherent fatty and epineural connective tissue and the whole nerve frozen and ground to a powder as described for the dog brain.

Analytical methods

For each tissue two pairs of duplicate samples of the frozen powder (100–200 mg.) were transferred to tared centrifuge tubes and the tubes reweighed. After the removal of acid-soluble P (I) and lipid P (II), as described by Schneider (1945), each pair of duplicate samples was submitted to either the Schmidt-Thannhauser or Schneider procedure as outlined in Table 2. One pair was hydrolysed for 18 hr. at 37° in N-KOH. The hydrolysate was divided into two portions, one of which was used for the determination of total P (III) and the other for the precipitation of the

Table 2. *Scheme outlining determinations*



Preparation of tissue

Estimations were done on the white matter and grey matter of the brains of six dogs and on the sciatic nerves of six cats. The animals were anaesthetized with nembutal, bled, and the brain or sciatic nerve removed as soon as possible after death. Samples of brain tissue (0·5–1 g.) were frozen in liquid N_2 and ground to a fine powder in a mortar. The sample of grey matter consisted of thin shavings of cortex from the cerebral hemispheres. The sample of white

DNA with 5N-HCl and 5% (w/v) TCA. The DNA was removed by centrifuging. One portion of the supernatant was used for the determination of total P (IV) and another for the determination of the ortho-P (V) by the modification of the method of Berenblum & Chain (1938) described by Ernster, Zetterström & Lindberg (1950). The precipitated DNA was extracted with 5% (w/v) TCA for 15 min. at 90°, as described by Schneider (1946). The total P was estimated in one portion of the extract (VI) and the DNA was estimated by ultraviolet absorption (VII) in another. Details

of this method are given in the Appendix. The residue remaining after the extraction of the DNA contained a negligibly small concentration of P.

The other pair of duplicate samples from which the acid-soluble P and lipid P had been removed was treated with 5% (w/v) TCA for 15 min. at 90°, as described by Schneider (1945). Portions of the TCA extract were used for the determination of DNA by the diphenylamine reaction of Dische (1930) (VIII), for the determination of PNA by the orcinol reaction of Mejbaum (1939) (IX), for the determination of total P (X), and for the determination of total nucleic acid by the method of ultraviolet absorption described in the Appendix (XI). A total P estimation was also done on the residue remaining after the TCA extraction (XII).

All fractions were made up in volumetric flasks, and portions containing 10–20 μg. P were taken for each P determination. Total P was estimated by the method of King (1932). The colour was read in a Coleman Universal spectrophotometer at 650 mμ. The diphenylamine and orcinol reactions were performed as described by Schneider (1945), the final readings being made at 600 and 655 mμ. respectively. Results are all presented in terms of the P content of the standard solutions.

RESULTS

Comparison of methods

Table 3 shows the concentration of acid-soluble P, lipid P and protein-bound P in the white matter and grey matter of dog brain and the sciatic nerve of the cat. These values agree in most respects with those reported for the brain and nerve of the monkey by Bodian & Dziewiatkowski (1950), and for the sciatic nerve of the guinea pig by Samuels *et al.* (1951). The concentration of lipid P in white matter is more than twice that in grey matter. The figures for the lipid P of white matter and grey matter of dog brain and cat sciatic nerve are of the same order as those reported for similar mammalian tissues (see Johnson, McNabb & Rossiter, 1948, 1949, for references). It will be noted that, as originally pointed out by Davidson & Waymouth (1944), the concentration of protein-bound P in white matter exceeds that in grey matter.

Table 4 compares the concentration of total nucleic acid determined by the Schmidt-Thannhauser method with that determined in the Schneider TCA extract by (*a*) the colour reactions, (*b*) the total P, and (*c*) the ultraviolet-absorption method. The values obtained by the Schmidt-Thannhauser method are all greater than those for the total P in the TCA extract and these, in turn, are greater than the values for the ultraviolet-absorption method. The greatest difference is in white matter and the least in grey matter. The values given by the colour reactions are also considerably higher than those given by the method of ultraviolet absorption.

Tables 5 and 6 give the values obtained for the concentration of DNA and PNA by a number of different methods. For all three tissues there is good agreement between the figures for DNA obtained by the Schmidt-Thannhauser method, the modification of this method described by Schneider (1946), and the ultraviolet-absorption method. The only values that do not agree are those given by the diphenylamine colour reaction of Dische (1930). These are higher for all three tissues.

It was with the PNA that the greatest differences were found (Table 6). The values for the concentration of PNA obtained by the Schmidt-Thannhauser

Table 3. *Distribution of phosphorus compounds in tissue from the nervous system*

(Mean ± S.E.M. of six determinations, expressed as mg. P/100 g. wet tissue.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve
Acid-soluble P	I	74·5 ± 4·2	79·5 ± 3·1	52·0 ± 2·1
Lipid P	II	333 ± 14	157 ± 6	308 ± 23
Protein-bound P	III	33·9 ± 2·4	24·6 ± 1·1	22·3 ± 1·0
Total P	I + II + III	441 ± 19	261 ± 10	382 ± 26

Table 4. *Total nucleic acid of tissue from nervous system*

(mg. P/100 g. wet tissue. Mean of six experiments ± S.E.M.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve	Measured
Schmidt & Thannhauser (1945)	III − V	31·3 ± 1·7	23·8 ± 0·6	20·1 ± 1·0	DNA + PNA + 'inositide P'
Schneider (1945), colour reactions	VIII + IX	21·1 ± 2·5	25·4 ± 1·2	14·9 ± 1·1	DNA + PNA + ?
Schneider (1945), phosphorus	X	18·8 ± 2·5	16·5 ± 0·3	14·0 ± 1·5	DNA + PNA + part of 'inositide P'
Ultraviolet absorption	XI	10·7 ± 0·5	15·2 ± 0·3	10·2 ± 0·5	DNA + PNA

method are all considerably greater than those obtained by the ultraviolet absorption method. The greatest difference is for white matter and the least is for grey matter. The values obtained by the orcinol colour reaction of Mejbaum (1939) are also slightly greater for all three tissues ($P<0{\cdot}01$, in each instance). Table 6 also gives the values of two extraction (XII), are greater than those found for the 'phosphoprotein' of Schmidt & Thannhauser (1945), i.e. the ortho-P liberated on hydrolysis in N-potassium hydroxide at 37° for 18 hr. (V). The difference is again greatest for white matter and least for grey matter, with the sciatic nerve being intermediate.

Table 5. *DNA of tissue from nervous system*

(mg. P/100 g. wet tissue. Mean of six experiments ± S.E.M.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve	Measured
Schmidt & Thannhauser (1945)	III – IV	6·1±0·2	4·5±0·2	4·8±0·2	DNA
Schmidt & Thannhauser (1945), modified by Schneider (1946)	VI	6·7±0·2	4·7±0·3	5·5±0·2	DNA
Schneider (1945), colour reaction of Dische (1930)	VIII	14·2±2·1	13·1±1·5	6·6±0·6	DNA + ?
Ultraviolet absorption	VII	5·8±0·4	4·9±0·4	5·4±0·4	DNA

Table 6. *PNA of tissue from nervous system*

(mg. P/100 g. wet tissue. Mean of six experiments ± S.E.M.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve	Measured
Schmidt & Thannhauser (1945)	IV – V	25·2±1·6	19·3±0·8	14·3±0·8	PNA + 'inositide P'
Schneider (1945), colour reaction of Mejbaum (1939)	IX	6·9±0·4	12·4±0·5	7·9±0·7	PNA + ?
Combination I of Schmidt & Thannhauser (1945), and Schneider (1945)	IV – XII	10·7±1·5	11·6±0·7	8·1±1·0	PNA + part of 'inositide P'
Combination II of Schmidt & Thannhauser (1945), and Schneider (1945)	X – VI	11·0±2·3	11·1±0·6	6·8±0·5	PNA + part of 'inositide P'
Ultraviolet absorption	XI – VII	4·9±0·2	10·3±0·4	4·7±0·5	PNA

Table 7. *'Phosphoprotein' of tissue from nervous system*

(mg. P/100 g. wet tissue. Mean of six experiments ± S.E.M.)

	Determination	Dog white matter	Dog grey matter	Cat sciatic nerve	Measured
'Phosphoprotein' of Schmidt & Thannhauser (1945)	V	2·6±0·2	2·4±0·2	2·2±0·2	PP ('phosphoprotein' phosphorus)
'Phosphoprotein' of Schneider (1945)	XII	15·3±1·2	7·7±0·6	10·2±0·9	PP + part of 'inositide P'
'Inositide P'	III – (V + XI)	20·6±1·7	8·3±0·4	10·0±1·0	'Inositide P'
'Inositide P'	(IV – V) – (XI – VII)	20·3	9·0	9·6	'Inositide P'

frequently used estimates of the concentration of PNA obtained by combining the procedures of Schmidt & Thannhauser (1945) and Schneider (1945). For white matter and peripheral nerve the figures are greater than those obtained with the ultraviolet absorption method, but for grey matter the differences are not statistically significant ($P=0{\cdot}2$ and $0{\cdot}3$, respectively).

Table 7 shows that the values found for the concentration of 'phosphoprotein' of Schneider (1945), i.e. the phosphorus remaining after the TCA

If it is assumed that the ultraviolet-absorption values are the more reliable, it is clear that the method of Schmidt & Thannhauser (1945), while giving a good estimate of the concentration of DNA, gives a gross overestimate of the concentration of PNA, especially for white matter and, to a lesser extent, for peripheral nerve. Both the colour reactions and the phosphorus estimations of Schneider (1945) also give results that are too high. A hint as to the cause of some of these discrepancies is that the values for the concentration of 'phos-

phoprotein' of Schneider (1945) are much greater than those obtained by the method of Schmidt & Thannhauser (1945), especially for white matter (Table 7). It is clear that much of the phosphorus estimated as 'phosphoprotein' by the former method is measured as nucleic acid, probably as PNA, by the latter. It was decided, therefore, to investigate the extraction of nucleic acid by the Schneider procedure.

Extraction of phosphorus by 5 % (w/v) TCA at 90°

The acid-soluble P and lipid P were removed from four pairs of duplicate samples of cat sciatic nerve and the residue was treated for periods of 5, 10, 15 or 30 min., with 5 % (w/v) TCA at 90° as described by Schneider (1945). Fig. 1 shows that after 15 min.

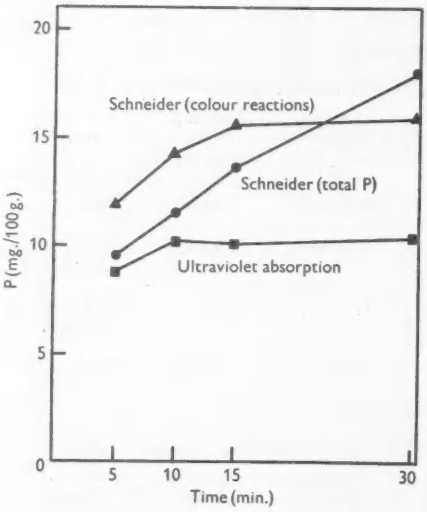


Fig. 1. Effect of time of heating with 5 % (w/v) TCA at 90°, on the values obtained for the concentration of total nucleic acid in cat sciatic nerve by three different methods. ▲—▲—▲, colour reactions of Schneider (1945); ●—●—●, total P of extract (Schneider, 1945); ■—■—■, ultraviolet absorption method.

there was little increase in the concentration of nucleic acid in the extract, as measured by the colour reactions (VIII + IX) or ultraviolet absorption (XI), but that the total P in the extract (X) increased with time. At 15 min. (the usual time of extraction in the Schneider procedure) the values were relatively the same as those presented in Table 3. The figure for the colour reactions is the greatest, followed by that for the total P and the ultraviolet absorption, in that order. With an increase in the length of the extraction time, more phosphorus was removed without any corresponding increase in the ultraviolet absorption, or the value given by the colour reactions. It is clear that some phosphorus compound not possessing the characteristic ultraviolet-absorption properties of nucleic acid or nucleotides was extracted by the TCA. There would thus appear to be no justification for the common practice of designating all phosphorus removed by extraction with TCA for 15 min. as nucleic acid P, and the phosphorus remaining in the residue after this time as 'phosphoprotein' P.

Fig. 2 gives the result of a similar experiment in which both the total P (X) and the ortho-P were measured in the extract and the total P (XII) was measured in the residue. Only a small fraction of the phosphorus of the extract was orthophosphate. In other experiments, in which the time of the extraction was prolonged (2 hr. or more), the residue P tended to zero and the ortho-P in the extract tended to the value found for the Schmidt-Thannhauser 'phosphoprotein' (V). After the usual extraction of 15 min. there was negligible ortho-P in the TCA extract.

Essentially similar results were obtained in a number of other experiments with cat sciatic nerve and with both white matter and grey matter of dog

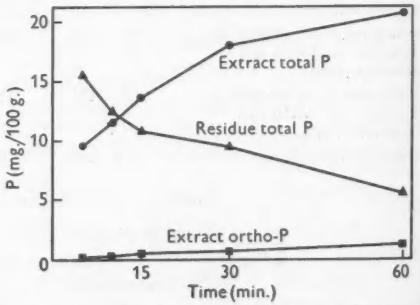


Fig. 2. Effect of time of heating with 5 % (w/v) TCA at 90° on the total P and ortho-P of the extract, and the total P of the residue of cat sciatic nerve from which the acid-soluble P and lipid P had previously been removed. ●—●—●, total P of extract; ■—■—■, ortho-P of extract; ▲—▲—▲, total P of residue.

brain. With white matter more phosphorus was extracted by the TCA and the time necessary for complete extraction was longer. At 15 min. the value for the concentration of nucleic acid calculated from total P was often of the same order as that calculated from the colour reactions. With grey matter, on the other hand, the values given by the colour reactions were greater than those calculated from total P. In no instance were we able to confirm the report of Samuels *et al.* (1951) that the concentration of nucleic acid determined by ultra-

violet absorption agreed with the phosphorus values. With other tissues (e.g. pancreas and spleen) the agreement was good.

When the residue remaining after treatment with 5% (w/v) TCA at 90° for 15 min. was subjected to the Schmidt-Thannhauser hydrolysis almost all the material passed into solution and only a further small quantity, containing negligible phosphorus, was precipitated when the solution was neutralized and TCA added to make the final concentration 5%. Table 8 shows that very little of the phosphorus of the residue appeared in the hydrolysate as ortho-phosphate. The concentration of ortho-P was found to be similar to that obtained for the 'phospho-protein' of Schmidt & Thannhauser (1945) (V) in a parallel experiment.

Table 8. *Residue phosphorus of cat brain after extraction with* 5% *(w/v) TCA for* 15 *min. at* 90°

(mg. P/100 g. wet tissue.)

	Experiment 1	Experiment 2
Total residue P (XII)	8·2	11·3
After Schmidt & Thannhauser (1945) hydrolysis:		
Ortho-P	3·2	2·1
Organic P	5·0	9·2
'Phosphoprotein' of Schmidt & Thannhauser (1945) (V)	3·5	2·3

Similar concentrations of ortho-P are thus obtained when tissue from the nervous system, from which the acid-soluble and lipid P has been removed, is (*a*) hydrolysed by the method of Schmidt & Thannhauser (1945), (*b*) subjected to prolonged extraction (longer than 2 hr.) with 5% (w/v) TCA at 90°, and (*c*) treated with 5% (w/v) TCA at 90° for 15 min. and the residue subjected to the Schmidt-Thannhauser hydrolysis. The origin of this phosphorus is obscure. Presumably it is derived from an acid-insoluble compound which, like casein, yields ortho-P on hydrolysis. This phosphorus represents the 'phosphoprotein' of Schmidt & Thannhauser (1945) and will be referred to as PP.

The phosphorus, some of which remains in the residue after extraction for 15 min. with 5% (w/v) TCA at 90°, and which is slowly removed by further extraction, is undoubtedly determined as PNA in the Schmidt-Thannhauser procedure and presumably causes the high values when PNA is determined by this method (Table 6). The substance responsible for these high PNA values interferes in the same way as the protein-bound metaphosphate of yeast (Wiame, 1947, 1949; Schimdt, Hecht & Thannhauser, 1946). Protein-bound metaphosphate was not found in animal tissues by Ebel (1949) or Davidson, Frazer & Hutchison (1951). However, a phosphorus compound with the requisite properties is the trypsin-resistant lipid-protein complex isolated from the white matter of calf brain by Folch & Le Baron (1951). This substance, which contains inositol diphosphate, is not removed from brain tissue by the usual ethanol-ether extraction, and Folch & Le Baron (1951) state that all of the phosphorus of this compound appears as PNA in the method of Schmidt & Thannhauser (1945). Folch (1951) states that this substance occurs chiefly in white matter of brain, and he believes it to be part of the classical 'neurokeratin' described by Kühne & Chittenden (1889) and thought to be a constituent of the myelin sheath of a mammalian nerve fibre. For further comments on the chemical nature of 'neurokeratin', see the paper by Folch & Lees (1951). The phosphorus of the protein-bound P (III) that cannot be accounted for either as nucleic acid or as PP will be referred to as 'inositide P'. It should be stressed that there is no evidence that all such phosphorus is present in brain as inositide, although it is shown (see below) that this type of phosphorus compound occurs in much greater concentration in white matter than in grey matter.

An estimate of the concentration of the 'inositide P' remaining after the removal of acid-soluble P and lipid P can be obtained by subtracting the sum of the values found for the PP (V) and the total nucleic acid determined by ultraviolet absorption (XI) from the protein-bound P (III). By subtracting the ultraviolet-absorption value for PNA (XI – VII) from the value obtained by the Schmidt-Thannhauser method (IV – V), another estimate of 'inositide P' can be obtained by a slightly different method.

Table 7 gives the concentration of 'inositide P' determined by each of these methods. By far the greatest concentration is in the white matter of dog brain, where 'inositide P' accounts for two-thirds of the protein-bound P. The concentration in grey matter and cat sciatic nerve is considerably lower.

Extraction of chromogenic material by 5% *(w/v) TCA at* 90°

Fig. 3 shows the results of an experiment similar to that represented in Fig. 1 in which the colour reactions of Dische (1930) and Mejbaum (1939) were carried out on TCA extracts of cat sciatic nerve. The extraction times were from 5 to 60 min. Although it is shown in Fig. 1 that the total nucleic acid determined by the colour reactions did not increase when the extraction time was increased from 15 to 30 min., Fig. 3 shows that after 15 min. the colour given by the diphenylamine reaction became less, and that given by the orcinol reaction became greater. These were consistent findings and were repeated in a number of similar experiments

with white matter and grey matter of dog brain as well as with cat sciatic nerve.

The lower values for the diphenylamine reaction with extraction times greater than 15 min. were obtained with tissues other than nerve or brain, although the higher values given by the orcinol reaction were peculiar to tissue from the nervous

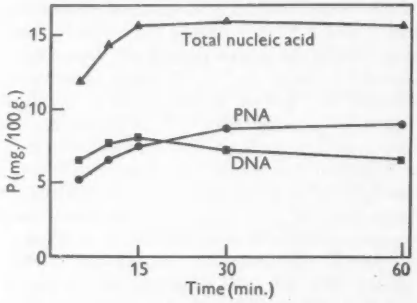


Fig. 3. Effect of time of heating with 5% (w/v) TCA on the values obtained for the concentration of total nucleic acid, DNA and PNA in cat sciatic nerve by the colour reactions. ▲—▲—▲, total nucleic acid by addition; ■—■—■, DNA by method of Dische (1930); ●—●—●, PNA by the method of Mejbaum (1939).

system. With extracts of brain or nerve the colour produced in the diphenylamine reaction was a blue-green rather than the clear blue of the DNA standard. With white matter the peak of the absorption curve of the colour complex was the same as that of the standard, but the curve was much flatter. With grey matter the curve was greatly distorted and the peak was shifted to lower wavelengths. When the reaction was carried out on DNA isolated by the Schmidt-Thannhauser method, the values obtained were similar to those given by total P (VI) or ultraviolet absorption (VII), and they were much lower than those obtained when the reaction was done on the Schneider TCA extract (VIII). The absorption characteristics of the colour complex given by the isolated DNA appeared normal. In the orcinol reaction the green colour obtained with brain extracts appeared the same as that given by the PNA standard, and the characteristics of the absorption curve of the colour complex were similar.

The results indicate that these two colour reactions are not suitable for estimating nucleic acid in TCA extracts of tissue from the nervous system.

DISCUSSION

It would thus appear that tissue from the nervous system contains at least two substances that interfere greatly with methods widely used for the determination of nucleic acids: (1) the 'inositide P' that is estimated as PNA by the method of Schmidt & Thannhauser (1945) and is measured, in part, as nucleic acid P by the method of Schneider (1945), and (2) chromogenic material that interferes with both the diphenylamine reaction of Dische (1930) and the orcinol reaction of Mejbaum (1939). Table 9 summarizes the substances that would be measured in each of the determinations outlined in 'Analytical methods'. In addition, some of the substances that would be measured in each of the estimates of total nucleic acid, DNA and PNA and 'phosphoprotein' given in Tables 4–7, can be found in the last column of the tables.

No attempt was made to determine whether a phosphorus compound similar to that which gives rise to the 'inositide P' occurs in tissues other than those of the nervous system. The experiments of Davidson *et al.* (1951) would suggest the possibility

Table 9. *Substances estimated in each of the determinations outlined in 'Analytical methods'*

Determination	Previously assumed	Estimated
I	Acid-soluble P	Acid-soluble P
II	Lipid P	Lipid P
III	DNA + PNA + 'phosphoprotein' of Schmidt & Thannhauser (1945)	DNA + PNA + PP + 'inositide P'
IV	PNA + 'phosphoprotein' of Schmidt & Thannhauser (1945)	PNA + PP + 'inositide P'
V	'Phosphoprotein' of Schmidt & Thannhauser (1945)	PP
VI	DNA of Schmidt & Thannhauser (1945)	DNA
VII	DNA by ultraviolet absorption	DNA
VIII	DNA by Dische (1930) diphenylamine reactions (Schneider, 1945)	DNA + ?
IX	PNA by Mejbaum (1939) orcinol reaction (Schneider, 1945)	PNA + ?
X	DNA + PNA by phosphorus (Schneider, 1945)	DNA + PNA + part of 'inositide'
XI	DNA + PNA by ultraviolet absorption	DNA + PNA
XII	'Phosphoprotein' of Schneider (1945)	PP + part of 'inositide P'

that smaller concentrations of such a compound may be present in liver. In most tissues the concentration of nucleic acid is considerably greater than in brain or nerve, and the Schneider 'phosphoprotein' (XII) represents a much smaller percentage of the total protein-bound P. The effect on the quantitative determination of nucleic acid would, therefore, be much less. However, Jeener (1949) and Davidson *et al.* (1951) have shown that after the administration of ^{32}P the specific activity of the Schmidt-Thannhauser PNA fraction of liver (our IV – V) is much greater than that of isolated PNA, even after the very active PP (our V) has been removed. These results suggest that liver contains small amounts of a metabolically active phosphorus compound that interfere with specific activity measurements made by the Schmidt-Thannhauser method. This interference is much greater in tissue of the nervous system. 'Inositide P' accounts for over 80% of the Schmidt-Thannhauser PNA (IV – V) in the white matter of dog brain (Table 5) and Strickland (1951), working in this laboratory, has shown that ^{32}P is incorporated into the PP and 'inositide P' of brain and nerve much more readily than into the DNA or PNA.

The gross differences between the estimates of the concentration of nucleic acids obtained by the methods of Schmidt & Thannhauser (1945), Schneider (1945) and by ultraviolet absorption are greatest in tissue of the nervous system. In a number of experiments with pancreas, spleen, thymus, white cells and reticulocytes, in which the same techniques and the same standards were used, fair agreement was obtained between the methods. This would strongly suggest that the differences observed for the nervous system are genuine. Since each of the three methods is dependent upon a different part of the nucleic acid molecule, it is at least theoretically possible that the lack of agreement is due to some fundamental difference in the structure of the nucleic acids of the nervous system. We are aware of no studies on the chemistry of nucleic acids isolated from the nervous system, but in view of recent studies on nucleic acid structure (i.e. Chargaff, 1951) such a possibility would appear to be unlikely.

Both Schneider (1946) and Tsuboi (1950) reported good agreement between various nucleic acid methods for a number of tissues, although it is apparent from the paper of Schneider (1946) that in rat brain the value for the concentration of total nucleic acid, determined by the Schmidt-Thannhauser method, is greater than that determined either by total P or by colour reactions done on the Schneider extract. The difference is due to an increase in the figure for PNA. Schneider (1945, 1946) also reported that for brain tissue the concentration of total nucleic acid in the TCA extract given by the colour reactions agreed with the figure given by phosphorus estimations. This agreement is fortuitous. The phosphorus figures are too high because of the presence of some 'inositide P', and the figures given by the colour reactions are too high because of interfering chromogenic material.

The results obtained by the ultraviolet absorption method for the concentration of nucleic acid in brain and nerve are much lower than the figures published by previous workers (Kossel, 1882; Berenblum, Chain & Heatley, 1939; Rosenthal & Drabkin, 1943; Davidson & Waymouth, 1944; Schmidt & Thannhauser, 1945; Schneider, 1945, 1946; Schneider & Klug, 1946; von Euler & Hahn, 1948; Bodian & Dziewiatkowski, 1950; Samuels *et al.* 1951). In Table 10 our results are compared with those obtained in the recent studies of Bodian & Dziewiatkowski (1950) and Samuels *et al.* (1951). As already pointed out, our results for acid-soluble and lipid P in the white matter and grey matter of dog brain and cat sciatic nerve agree well with the published figures for monkey brain and guinea-pig nerve, but our figures for total nucleic acid, DNA, and 'phosphoprotein' are considerably lower.

It is of interest to note that the concentration of total nucleic acid in grey matter is considerably greater than that in white matter. White matter and grey matter of dog brain and cat sciatic nerve all contain similar concentrations of DNA, but there is a much higher concentration of PNA in grey matter. This is not surprising, since grey matter contains many more nerve cell bodies. These contain abundant Nissl material, which is believed to be rich in PNA. White matter and grey matter of dog brain and cat sciatic nerve also contain a similar concentration of PP (V).

The finding of Bodian & Dziewiatkowski (1950) that white matter contains more 'phosphoprotein' (our XII) than grey matter is confirmed, although the values for dog brain reported in Table 7 are somewhat lower, both for white matter and for grey matter, than the figures given by these workers for monkey brain. The high concentration of phosphorus in the 'phosphoprotein' fraction of white matter is due to the much greater concentration of 'inositide P', much of which remains in this fraction after the TCA extraction. The high concentration of 'inositide P' in white matter, taken in conjunction with our further finding that this substance disappears from a peripheral nerve during Wallerian degeneration, even although the concentration of nucleic acid increases (Logan, Mannell & Rossiter, 1952), gives added weight to the suggestion of Folch (1951) that the 'inositide P' is derived from the 'neurokeratin' of the myelin sheath. It is this substance and not nucleic acid that contributes to the high protein-bound P found in white matter by Davidson & Waymouth (1944).

Table 10. *Phosphorus compounds in tissue from nervous system. Comparison of present results with those in recent literature*

(mg. P/100 g. wet tissue ± S.E.M.)

Observer	No. of animals	Tissue	Acid-soluble P	Lipid P	Total nucleic acid	DNA	PNA	PP	'Inositide P'
				(1) *White matter*					
Bodian & Dziewiatkowski (1950)	4 monkeys	Corpus callosum	50·5 ± 2·1	377 ± 12	18·8 (*a*)	12·4 ± 1·6 (*b*)	6·4 ± 2·2 (*c*)	23·2 ± 2·6 (*d*)	—
Present results	6 dogs	Chiefly corpus callosum	74·5 ± 4·2	333 ± 14	11·5 (*e*)	6·3 ± 0·4 (*f*)	5·3 ± 0·2 (*g*)	2·6 ± 0·2 (*h*)	19·8 ± 1·7 (*i*)
				(2) *Grey matter*					
Bodian & Dziewiatkowski (1950)	4 monkeys	Caudate nucleus	86·0 ± 2·1	185 ± 12	21·5 (*a*)	9·6 ± 1·6 (*b*)	11·5 ± 2·2 (*c*)	12·1 ± 2·6 (*d*)	—
Present results	6 dogs	Grey cerebral cortex	79·5 ± 3·1	157 ± 6	16·4 (*e*)	5·3 ± 0·4 (*f*)	11·1 ± 0·4 (*g*)	2·4 ± 0·2 (*h*)	7·5 ± 0·4 (*i*)
				(3) *Peripheral nerve*					
Bodian & Dziewiatkowski (1950)	5 monkeys	Brachial and sciatic nerves	34·5 ± 3·7	328 ± 14	—	—	—	—	–
Samuels *et al.* (1951)	26 guinea pigs	Sciatic nerve	50 ± 5	313 ± 45	19·4 ± 5 (*j*)	—	—	27·0 ± 5 (*d*)	—
Present results	6 cats	Sciatic nerve	52·0 ± 2·1	308 ± 23	11·0 ± 0·5 (*e*)	5·8 ± 0·4 (*f*)	5·1 ± 0·5 (*g*)	2·2 ± 0·2 (*h*)	9·2 ± 1·0 (*i*)

(*a*) By colour reactions (our VIII + IX).
(*b*) By Dische (1930) diphenylamine reaction (our VIII).
(*c*) By Mejbaum (1939) orcinol reaction (our IX).
(*d*) 'Residue P' after TCA extraction (our XII).
(*e*) By ultraviolet absorption (XI).
(*f*) By ultraviolet absorption (VII).
(*g*) By ultraviolet absorption (XI – VII).
(*h*) By Schmidt & Thannhauser (1945) (V).
(*i*) By calculation [III – (V + XI)].
(*j*) By P estimation according to Schneider (1945) (our X).

SUMMARY

1. The concentration of acid-soluble phosphorus, lipid phosphorus and protein-bound phosphorus was determined in the white matter and the grey matter of dog brain and in the sciatic nerve of the cat. In addition, the concentration of total nucleic acid, deoxypentosenucleic acid (DNA), pentosenucleic acid (PNA) and 'phosphoprotein' was determined by the methods of Schmidt & Thannhauser (1945), Schneider (1945), and by an ultraviolet absorption method.

2. The figures for total nucleic acid, DNA and PNA given by the method of Schneider (1945), and those for total nucleic acid and PNA given by the method of Schmidt & Thannhauser (1945), were greater than the corresponding figures given by the ultraviolet absorption method. With other tissues there was good agreement between the three methods.

3. An investigation of the causes of these discrepancies revealed that tissue of the nervous system contains (*a*) a phosphorus compound, probably the inositol-containing trypsin-resistant lipid-protein complex of Folch & Le Baron (1951), which is estimated as PNA by the method of Schmidt & Thannhauser (1945) and is measured, in part, as nucleic acid phosphorus in the method of Schneider (1945), and (*b*) chromogenic material that interferes with the colour reactions in the method of Schneider (1945).

4. The values obtained for the concentration of nucleic acid in tissue from the nervous system by the method of ultraviolet absorption are considerably lower than those previously published in the literature. The concentration of DNA is of the same order in white matter and grey matter of dog brain and in cat sciatic nerve, but the concentration of PNA is much greater in the grey matter.

5. The concentration of 'phosphoprotein' (PP) is of the same order in white matter and grey matter of dog brain and in cat sciatic nerve, but the concentration of 'inositide P' is much greater in white matter.

6. Some comments on the determination of DNA and PNA in tissue from the nervous system by the method of ultraviolet absorption are given in the Appendix.

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APPENDIX

A Note on the Determination of Deoxypentosenucleic Acid and Pentosenucleic Acid in Tissue from the Nervous System by Ultraviolet Absorption

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Schneider (1945) introduced the convenient method of extracting nucleic acids from tissue with 5% (w/v) TCA. Such an extract, which contains minimal quantities of protein, may be used for the determination of nucleic acid by ultraviolet absorption.

The tissue samples were prepared as described in 'Analytical methods'. The extinction of (*a*) the TCA extract containing the DNA (VII) and (*b*) the TCA extract containing the total nucleic acid (XI) was determined in a Beckman Model DU quartz spectrophotometer at 268·5 mμ. The concentration of nucleic acid (in mg. P/100 g. wet tissue) for both DNA and total nucleic acid was calculated on the assumption that both DNA and PNA have an ε (P) of 9850 at this wavelength (see below). The concentration of PNA was determined by difference.

The procedure presented certain difficulties worth recording.

(1) *Change in absorption characteristics of standards on heating for* 15 *min. at* 90° *in* 5% *(w/v) TCA*

After heating in 5% (w/v) TCA at 90° for 15 min. the absorption maximum of the standard DNA was shifted from 259 to 267 mμ. and the ε (P) at maximum absorption increased from 7300 to 9900 (Fig. 4). After neutralizing and buffering in 0·1 M-phosphate at pH 7 the absorption maximum returned to 263 mμ., but the extinction remained high. The changes in the standard PNA were in the same direction, but much less marked. After neutralizing and buffering at pH 7 the absorption maximum returned to

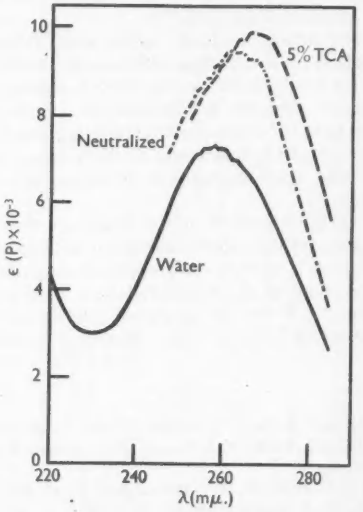

Fig. 4. Ultraviolet absorption of standard DNA. ——, in water; — — —, after heating with 5% (w/v) TCA at 90° for 15 min.; — · — ·, after heating with TCA as above, neutralizing and buffering in 0·1 M-phosphate at pH 7.

Table 11. *Effect of heating standard preparations of DNA and PNA for* 15 *min. at* 90° *in* 5% *(w/v) TCA*

	Standards used		Calculated for data of Tsuboi (1950)	
	$\lambda_{max.}$ (mμ.)	ε (P) at $\lambda_{max.}$	$\lambda_{max.}$ (mμ.)	ε (P) at $\lambda_{max.}$
DNA:				
In distilled water	259	7300	259	6900
After heating for 15 min. at 90° in 5% (w/v) TCA	267	9900	—	—
As above, but neutralized and buffered in 0·1 M-phosphate at pH 7·0	263	9500	262	9600*
PNA:				
In distilled water	258	9300	259	8300
After heating for 15 min. at 90° in 5% (w/v) TCA	261	10700	—	—
As above, but neutralized and buffered in 0·1 M-phosphate at pH 7·0	259	11000	259	10000*

* Heated for 20 min. at 90° in 5% (w/v) TCA. Readings at 260 mμ.

259 mμ. (Table 11). Similar changes in the ε (P) and $\lambda_{max.}$ of the standard DNA and PNA solutions were observed after heating in 6% (w/v) $HClO_4$ at 90° for 15 min. Other workers have described changes in the ultraviolet absorption characteristics of nucleic acids after acid, alkaline, or enzymic hydrolysis (Warren & Chanutin, 1948; Tsuboi, 1950; Little & Butler, 1951).

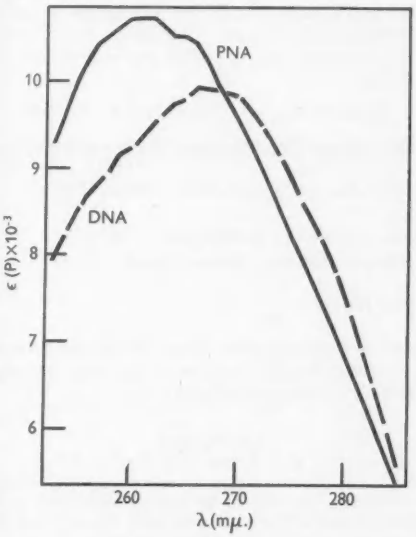

Fig. 5. Ultraviolet absorption of standard DNA and standard PNA after heating with 5% (w/v) TCA at 90° for 15 min. — — —, DNA; ——, PNA.

Tsuboi (1950) reported similar changes in the $\lambda_{max.}$ and ε of DNA and PNA solutions. Table 11 shows that the wavelength of maximum absorption, and the ε (P) of both the standard DNA and the standard PNA after treatment with TCA and neutralization, agree with results obtained by Tsuboi (1950).

The absorption curves of equal concentrations (on a P basis) of DNA and PNA were found to intersect at 268·5 mμ. when the measurements were made after heating in TCA without neutralization (Fig. 5). Accordingly, routine measurements were made at this wavelength where both the DNA and PNA have an ε (P) of 9850. This wavelength also has the added advantage that the absorption due to the TCA is much less than at 260 mμ. (see below).

(2) *Ultraviolet absorption of TCA*

Fig. 6 shows the great ultraviolet absorption of 5% (w/v) TCA. On the same graph is plotted the extinction of PNA (2 μg. P/ml.). It can be seen that at 260 mμ. the extinction due to the TCA is of the same order as that due to the nucleic acid. As a result, all reference blanks and standards must contain exactly the same concentration of TCA as does the test solution. In addition, the method suffers from the disadvantage that heating at 90° for 15 min. causes considerable destruction of TCA (Table 12), the TCA being broken down according to the equation

$$CCl_3COOH \rightarrow CHCl_3 + CO_2.$$

Because of this, the blanks and standards must be heated for exactly the same length of time as the test samples. Various attempts were made to obviate this difficulty. The TCA could be removed with solvents such as ether or ethyl acetate, but this was time-consuming and in samples of

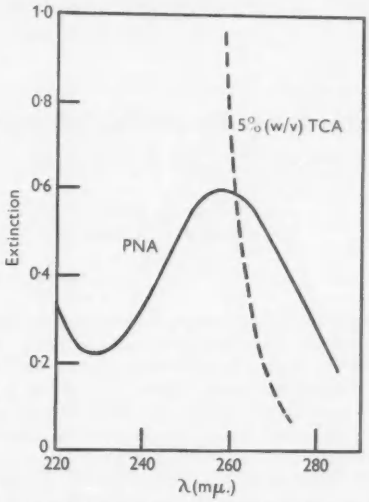

Fig. 6. Ultraviolet absorption of TCA and standard PNA. — — —, 5% (w/v) TCA; ——, PNA (2 μg. P/ml.).

biological tissue emulsions proved troublesome. $HClO_4$ (6%, w/v), which is free from interfering ultraviolet absorption, was found to extract the nucleic acids. However, when $HClO_4$ was used with tissue from the nervous system, the extracts absorbed strongly in the region of 274–276 mμ., presumably due to protein or protein degradation products. Experiments with casein showed that, when the 5% (w/v) TCA was replaced by 6% (w/v) $HClO_4$, more than three times the amount of material absorbing at 274–276 mμ. was extracted.

Table 12. *Extinction at 269 mμ. of 5% (w/v) TCA heated at 90°*

Heating time (min.)	$E^{5\%}_{1\,cm.}$ at 269 mμ.	
	Exp. 1	Exp. 2
0	0·162	0·162
5	0·158	0·157
10	0·150	0·150
15	0·135	0·132
30	0·113	0·114
60	0·092	0·090

As a routine, therefore, no attempt was made to remove the TCA. At 268·5 mμ. the proportionate effect due to any slight difference between the concentration of TCA in the test solution and in the reference blank is much less than at 260 mμ. (Fig. 6).

(3) *Extraction of nucleic acids*

Fig. 1 shows that the absorption at 268·5 mμ. of a TCA extract of cat sciatic nerve was not significantly greater when the extraction time was increased from 15 to 30 min. Similar results were obtained with white matter and grey matter of dog brain. However, after prolonged heating with TCA (2 hr.) a slight increase in the absorption at 268·5 mμ. was observed. When the residue remaining after a 15 min. extraction with TCA was extracted for a further 105 min., the extract had a slight absorption at 268 mμ., but the maximum absorption was at 274–275 mμ., presumably due to protein degradation products.

In the standard 15 min. extract of brain or nerve there was no suggestion of a secondary peak at 274–276 mμ. as was observed with 6% (w/v) $HClO_4$. It is felt that interference due to protein degradation products in samples heated in 5% (w/v) TCA for no longer than 15 min. is minimal.

Chemical Studies of Peripheral Nerve During Wallerian Degeneration

3. NUCLEIC ACIDS AND OTHER PROTEIN-BOUND PHOSPHORUS COMPOUNDS

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This paper describes changes in the concentration of nucleic acids and other protein-bound phosphorus compounds in the sciatic nerve of the cat after nerve section or nerve crush. If a nerve is cut (neurotmesis) that portion of the nerve peripheral to the point of section undergoes the familiar series of changes known as Wallerian degeneration. If the nerve is crushed, a procedure that interrupts the axons and myelin sheaths but leaves the connective tissue sheaths intact (axonotmesis), the nerve degenerates as before. After a short latent period the degenerating peripheral segment is re-innervated by axon tips from the intact central stump. There is thus a regeneration superimposed upon the degeneration. In previous publications from this laboratory changes in the concentration of lipids after nerve section or nerve crush have been described (Johnson, McNabb & Rossiter, 1949; Burt, McNabb & Rossiter, 1950).

While this work was in progress, papers by Bodian & Dziewiatkowski (1950) and Samuels *et al.* (1951) appeared giving some figures for protein-bound phosphorus compounds in peripheral nerve during Wallerian degeneration. By using the method of ultraviolet absorption described in the preceding paper (Logan, Mannell & Rossiter, 1952), a more reliable estimate of the concentration of total nucleic acid in a degenerating peripheral nerve has been obtained. In addition, the changes that occur in the concentration of deoxypentosenucleic acid (DNA), pentosenucleic acid (PNA), 'phosphoprotein' (PP) and 'inositide P' after nerve section or nerve crush have been described for the first time. The figures given for 'inositide P' probably represent the phosphorus of the inositol-containing trypsin-resistant lipid-protein complex described by Folch & Le Baron (1951) and thought by Dr Jordi Folch to be part of the 'neurokeratin' of Kühne & Chittenden (1889).

METHODS

The right sciatic nerves of fifty-two cats were either sectioned or crushed at the level of the greater trochanter of the femur. Details of the operations have already been described (Johnson *et al.* 1949; Burt *et al.* 1950). No attempt was made to control the age, sex or weight of the animals. After intervals of time varying from 2 to 600 days the animals were killed, and the segment of nerve distal to the site of the operation was removed. At the same time, a similar length of left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and epineural connective tissue, weighed, and the phosphorus compounds were estimated as described in the preceding paper (Logan *et al.* 1952). The nucleic acids were estimated by ultraviolet absorption.

Because of the great increase in the wet weight of degenerating and regenerating nerves, the results have all been expressed in terms of the fresh weight of a similar length of the intact nerve of the opposite side. This is equivalent to expressing the results in terms of the fresh weight of the nerve before it had been sectioned or crushed, i.e. at zero time. All analyses were done in duplicate.

RESULTS

Nerve section

Table 1 shows that 16 days after nerve section there was an increase in the concentration of acid-soluble P and protein-bound P, i.e. phosphorus remaining after the removal of acid-soluble P and lipid P, and a decrease in the concentration of lipid P and total P. Table 2 shows that the increase in the concentration of protein-bound P was due to an increase (by a

factor of 3 to 4) in the concentration of nucleic acid. The concentration of both DNA and PNA increased, but the increase in the concentration of PNA was greater than that of DNA, causing a rise in the PNA/DNA ratio. Neither PP nor 'inositide P' contributed to the rise in the protein-bound P. After 16 days the mean concentration of PP was less than that in the control nerves. The mean concentration of 'inositide P' was also less than that in the control nerves, although the difference is not significant statistically ($P = 0{\cdot}2$–$0{\cdot}3$). This is no doubt due to the lack of precision in the 'inositide P' determination. 'Inositide P' is obtained by difference from three individual determinations. After 32 days, however, the mean concentration of 'inositide P' in the degenerating nerves was very much less than that in the control nerves and the difference is statistically significant. The standard errors given in Tables 1 and 2 indicate the degree of reproducibility to be expected with different animals.

Figs. 1–5 show the changes in the concentration of the various phosphorus compounds at different times after nerve section. The concentration of acid-soluble P (Fig. 1) increased, reaching a maximum at 8–16 days and then decreased to values well below those of the control nerves. As was found in the previous study (Johnson *et al.* 1949), the concentration of lipid (Fig. 2) did not change greatly during the first 8 days and then decreased rapidly during the period 8–32 days.

Table 1. *Phosphorus compounds in sciatic nerve of the cat* 16 *days after nerve section or nerve crush*

(Results are expressed as mg. P/100 g. wet tissue. No. of animals is stated in parentheses under each result. *P* gives the significance of the difference between values for experimental and control nerves.)

	Control		16 days after nerve section			16 days after nerve crush		
Estimation	Mean	S.E.M.	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*
Acid-soluble P	52·2 (42)	± 0·7	62·1 (6)	± 2·1	<0·01	64·9 (5)	± 2·5	<0·01
Lipid P	310 (50)	± 5·6	156 (6)	± 20	<0·01	149 (5)	± 14	<0·01
Protein-bound P	19·4 (52)	± 0·5	41·7 (6)	± 1·2	<0·01	41·5 (5)	± 2·2	<0·01
Total P (by addition)	380·8 (42)	± 5·8	259·7 (6)	± 33·8	<0·01	254·9 (5)	± 11·7	<0·01

Table 2. *Protein-bound* P *in sciatic nerve of the cat* 16 *days after nerve section or nerve crush*

(Results are expressed as mg. P/100 g. wet tissue. No. of animals is stated in parentheses under each result. *P* gives the significance of the difference between values for experimental and control nerves.)

	Control		16 days after nerve section			16 days after nerve crush		
Estimation	Mean	S.E.M.	Mean	S.E.M.	*P*	Mean	S.E.M.	*P*
Total protein-bound P	19·4 (52)	± 0·5	41·7 (6)	± 1·2	<0·01	41·5 (5)	± 2·2	<0·01
Total nucleic acid	8·7 (52)	± 0·2	32·9 (6)	± 1·1	<0·01	34·5 (5)	± 2·3	<0·01
DNA	4·8 (52)	± 0·1	14·4 (6)	± 0·9	<0·01	13·4 (5)	± 0·5	<0·01
PNA	3·9 (52)	± 0·1	18·5 (6)	± 0·8	<0·01	21·1 (5)	± 2·4	<0·01
PP	1·8 (51)	± 0·1	1·0 (6)	± 0·1	<0·01	1·2 (5)	± 0·1	<0·01
'Inositide P'	9·0 (51)	± 0·4	7·7 (6)	± 0·8	<0·3	5·8 (5)	± 1·2	<0·02
PNA/DNA ratio	0·9 (52)		1·3 (6)			1·6 (5)		

Fig. 3 shows that the concentration of protein-bound P rose to a maximum at 16 days and then fell slowly throughout the course of the degeneration. The rise, which was apparent after only 2 days of degeneration, was due to an increase in the concentration of nucleic acid. There was a steady fall in the concentration of PP. The concentration of 'inositide P', like the concentration of lipid P, did not change greatly in the first 8 days and then fell sharply in the period 8–32 days.

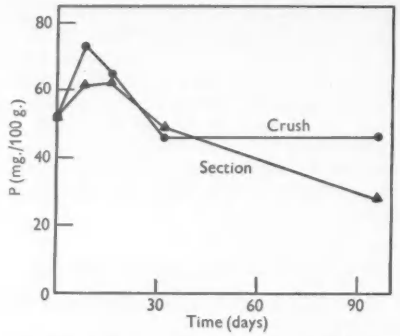

Fig. 1. Acid-soluble P of cat sciatic nerve after nerve section (▲—▲) or nerve crush (●—●). Each point represents the mean for three or more animals.

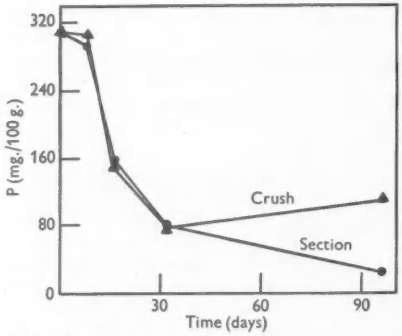

Fig. 2. Lipid P of cat sciatic nerve after nerve section (●—●) or nerve crush (▲—▲). Each point represents the mean for three or more animals.

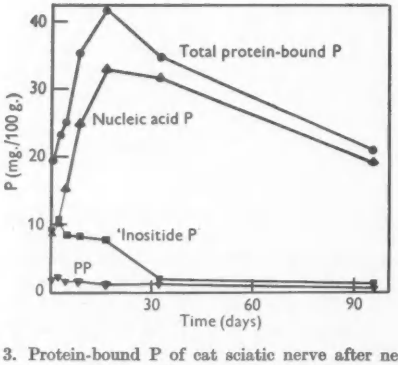

Fig. 3. Protein-bound P of cat sciatic nerve after nerve section. ●—●, total protein-bound P; ▲—▲, nucleic acid P; ■—■, 'inositide P'; ▼—▼, PP. Each point represents the mean for three or more animals.

Fig. 4 gives the concentrations of DNA and PNA. Both nucleic acids increased in concentration, but PNA increased more rapidly than DNA, so that by 32 days the PNA/DNA ratio had increased from 0·9 to 2·0 (Fig. 5). After 32 days the PNA/DNA ratio fell gradually.

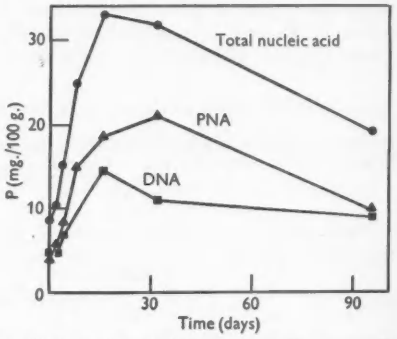

Fig. 4. Nucleic acids of cat sciatic nerve after nerve section. ●—●, total nucleic acid; ■—■, DNA; ▲—▲, PNA. Each point represents the mean for three or more animals.

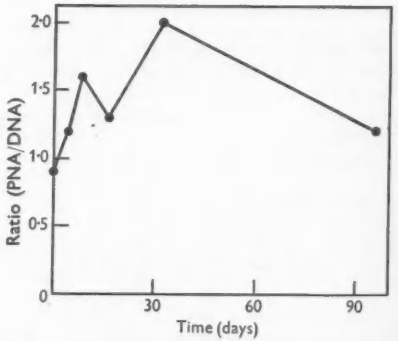

Fig. 5. Ratio of PNA to DNA in cat sciatic nerve after nerve section. Each point represents the mean for three or more animals.

Nerve crush

Tables 1 and 2 show that 16 days after nerve crush the phosphorus compounds changed in the same direction and to the same extent as after nerve section. None of the figures for the crushed nerves is significantly different from that for the corresponding substance in the sectioned nerves.

By 96 days signs of regeneration began to appear in the crushed nerves. The concentration of acid-soluble P (Fig. 1) and lipid P (Fig. 2) was greater than that in the sectioned nerves ($P < 0{\cdot}01$, in both instances). With much longer periods of regenera-

tion the concentration of total P and lipid P continued to increase (Fig. 6), and the concentration of protein-bound P continued to fall. Fig. 7 shows that the fall in protein-bound P was due to a fall in the concentration of nucleic acid. By 96 days the concentration of both PP and 'inositide P' had commenced to increase, even although the concentration of protein-bound P was still falling. Both DNA and PNA contributed to the fall in the concentration of nucleic acid, but the PNA decreased more rapidly than the DNA, so that the PNA/DNA ratio, which in the period 8–32 days was greatly in excess of the figure for the control nerves, fell steadily, reaching a value below that of the controls by 250 days (Fig. 8).

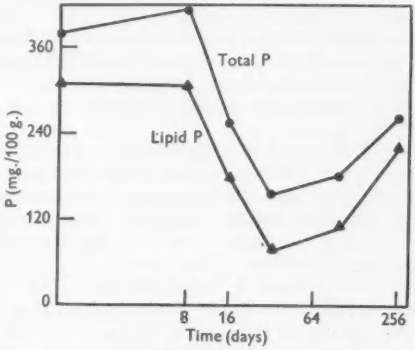

Fig. 6. Total P (●—●) and lipid P (▲—▲) of cat sciatic nerve after nerve crush. Each point represents the mean for three or more animals.

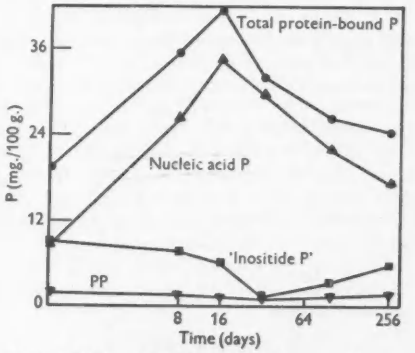

Fig. 7. Protein-bound P of cat sciatic nerve after nerve crush. ●—●, total protein-bound P; ▲—▲, nucleic acid P; ■—■, 'inositide P'; ▼—▼, PP. Each point represents the mean for three or more animals.

After 250 days, at a time when the acid-soluble P and PP had returned to normal, the concentration of both lipid P and 'inositide P' was still considerably less than that in the control nerves and the concentration of nucleic acid, especially of DNA, was greater. In four additional animals the nerves were crushed and then allowed to regenerate for 550–600 days after the operation. Even after this length of time the concentration of lipid P and 'inositide P' was still less than that in the control nerves of the opposite side and the concentration of nucleic acid was still greater.

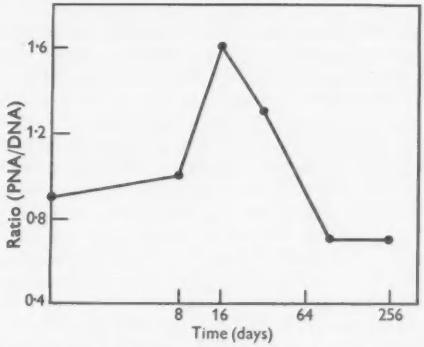

Fig. 8. Ratio of PNA to DNA in cat sciatic nerve after nerve crush. Each point represents the mean of three or more animals.

DISCUSSION

When a peripheral nerve is sectioned or crushed that portion of the nerve distal to the site of the lesion degenerates. The axon disintegrates and the myelin sheath at first fragments and ultimately is destroyed. The Schwann cells proliferate and the endoneurium and neurilemma thicken to form a Schwann tube, which is able to receive a growing axon tip from the intact central stump. The degeneration is, in a sense, a preparation for regeneration.

The sharp fall in the concentration of lipid P during the period 8–32 days after either type of operation is most likely the result of the destruction of the myelin sheath (Johnson, McNabb & Rossiter, 1950). The finding that the 'inositide P' also disappears at this time is of interest. If this fraction represents the lipid-protein complex of Folch & Le Baron (1951) and is part of the classical 'neurokeratin', the observation provides good evidence that 'neurokeratin' is a constituent of the myelin sheath of a peripheral nerve fibre. This view is strengthened by the further finding that during the deposition of myelin in nerves regenerating after a crush, the 'inositide P' returns to the nerve at the same time as the lipid P.

The rise in the concentration of nucleic acid, reaching a peak 16–32 days after nerve section or

nerve crush, is probably accounted for by the increase in the number of Schwann cells and endoneurial cells (chiefly fibrocytes and macrophages) that takes place during this time (Young, 1942; Abercrombie & Johnson, 1946). If, as has been suggested, the mean quantity of DNA per diploid cell is constant for any one species (see Davidson, Leslie & White, 1951, for references), it is apparent that the total amount of DNA in a nerve is an index of the number of cell nuclei present. The curve representing the change in the concentration of DNA with time (Fig. 4) is of the same general form as that reported by Abercrombie & Johnson (1946) for the change in the total number of cell nuclei in the degenerating sciatic nerve of the rabbit. Quantitatively the curves are different, but this is not surprising when the many differences between the two sets of experiments are taken into consideration. Our experiments were with cats, not rabbits, and the chemical determination gives an estimate of the number of cell nuclei throughout the whole length of the nerve rather than the number at one selected level. Also it is now known that the extent of the increase in the total number of cell nuclei in a peripheral nerve during Wallerian degeneration depends upon the size and the degree of myelination of the constituent fibres (Joseph, 1947, 1948; Thomas, 1948).

The concentration of PNA gives an indication of the total mass of cytoplasm within the nerve. During the period of cellular proliferation the PNA/DNA ratio, i.e. the concentration of PNA per cell, is considerably greater than that in the control nerves. At this time an increase in the amount of Schwann cell cytoplasm can be demonstrated histologically (Young, 1942). There is also an increase in the concentration of acid-soluble P.

The increase in the concentration of nucleic acid is statistically significant as early as 2 days after the operation, yet even at 8 days, when the concentration of nucleic acid is greatly in excess of that in the control nerves, the concentration of lipid P and 'inositide P' is not significantly decreased. This would indicate that the cellular proliferation commences well in advance of the chemical degradation of the myelin sheath, although, with the degeneration of the axon in the first few days after nerve section, there is a physical destruction of the myelin sheath, with segmentation and ovoid formation (Young, 1945; Johnson *et al.* 1950).

In the sectioned nerves there was no post-mortem or functional evidence of regeneration 96 days after the operation, and in other animals similarly treated this absence of regeneration was confirmed microscopically. In the crushed nerves, on the other hand, myelination had spread throughout the length of the nerve and function appeared normal. Yet even after 600 days, neither the lipid P nor the 'inositide P' had returned to normal. At this time the regenerating nerves still contained more nucleic acid than the control nerves of the opposite side. This finding is of interest in view of the slow restitution of fibre pattern (Gutman & Sanders, 1943; Sanders, 1948) and full physiological activity (Berry, Grundfest & Hinsey, 1944; Sanders & Whitteridge, 1946; Erlanger & Schoepfle, 1946) in regenerating nerves.

The figures for the concentration of phosphorus compounds in the control nerves agree well with those reported in the previous paper (Logan *et al.* 1952) for a smaller series of normal cat sciatic nerves.

May (1930), Bodian & Dziewiatkowski (1950) and Samuels *et al.* (1951) have studied the distribution of phosphorus compounds in peripheral nerves during Wallerian degeneration. Comparison of our results with those of May (1930) is of little value because of the great differences in the analytical techniques employed. Bodian & Dziewiatkowski (1950) reported a fall in the concentration of lipid P and a slight increase (of the order of 18 %) in the concentration of protein-bound P 7–23 days after section in monkey nerves. Because the 'inositide P' accounts for such a high percentage of the protein-bound P of the control nerves and the concentration of 'inositide P' falls while that of nucleic acid rises during Wallerian degeneration, the percentage increase in the concentration of nucleic acid would be much greater than that of the protein-bound P. In our experiments the protein-bound P of cat sciatic nerve increased by 116 % in 16 days, whereas the concentration of nucleic acid increased by 280 %. Samuels *et al.* (1951), using the method of Schneider (1945), also reported an increase in the concentration of nucleic acid (112 %) in guinea-pig nerves 16 days after section. For the reasons given by Logan *et al.* (1952), the figures of Samuels *et al.* (1951) for nucleic acid, especially those for the control nerves, are probably too high.

SUMMARY

1. The concentration of acid-soluble phosphorus, lipid phosphorus, protein-bound phosphorus, total nucleic acid, deoxypentosenucleic acid (DNA), pentosenucleic acid (PNA), phosphoprotein (PP) and 'inositide phosphorus' was determined in the sciatic nerve of the cat at intervals of time from 2 to 96 days after nerve section (neurotmesis) and 8–600 days after nerve crush (axonotmesis).

2. The acid-soluble phosphorus increased to a maximum at 8–16 days after either nerve section or nerve crush, returning to values not significantly different from those of the control nerves by 32 days. The acid-soluble phosphorus then remained normal in the crushed nerves, but fell to lower values in the sectioned nerves.

3. The lipid phosphorus changed little during the first 8 days and then decreased rapidly during the period 8–32 days after either nerve section or nerve crush. In the sectioned nerves the lipid phosphorus fell still further, whereas in the crushed nerves it had commenced to increase by 96 days and continued to increase throughout the period of regeneration.

4. The concentration of total nucleic acid increased after either nerve section or nerve crush, reaching a maximum in 16 days. Thereafter the concentration of nucleic acid fell in both the sectioned and crushed nerves. After each type of operation the PNA increased more rapidly than the DNA, so that there was an increase in the PNA/DNA ratio.

5. The PP fell throughout the whole course of the degeneration in the sectioned nerves. In the crushed nerves it had commenced to rise by 96 days, and by 250 days it approximated the concentration in the control nerves.

6. The 'inositide phosphorus' followed a course similar to that of the lipid phosphorus. After either type of operation there was little change during the first 8 days, followed by a rapid fall during the period 8–32 days. In the sectioned nerves the 'inositide phosphorus' fell still further, whereas in the crushed nerves it had commenced to increase by 96 days and continued to rise during the entire period of study.

7. The increase in the concentration of nucleic acid and the decrease in the concentration of lipid phosphorus and 'inositide phosphorus' still persisted 600 days after nerve crush.

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A Study of the Amino-acid Complexes Formed by Metals of Group II of the Periodic Classification

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Albert (1950) published a study of the formation of complexes between a number of metals and α-amino-acids containing only two ionizing groups. The mathematical treatment of the results from potentiometric titrations enabled the stabilities of the metal complexes to be calculated. Using his potentiometric method all the metals of Group II of the periodic classification were studied in their relation to glycine, its derivatives and other α-amino-acids with only two ionizing groups. In this paper an attempt has been made to study the effect on complex formation of (*a*) the nature of the metal, and (*b*) substitution in the amino-acid. An attempt was also made to correlate the results obtained by potentiometry with the solubility studies of Monk (1951*a*, *b*).

EXPERIMENTAL

Materials. The following salts were used in 0·01 M-solution: $BaCl_2.6H_2O$; $SrCl_2$; $CaCl_2$; $MgSO_4.7H_2O$; $BeSO_4.4H_2O$; $ZnSO_4.7H_2O$; $3CdSO_4.8H_2O$; $Hg(NO_3)_2$; $CoCl_2.5H_2O$; $NiCl_2.6H_2O$; $Pb(NO_3)_2$. $Hg(NO_3)_2$ was made up in 0·025 M-HNO_3 and excess acid back-titrated in the presence of the amino-acid investigated. $AgNO_3$ was used in 0·02 M-solution. All salts were of A.R. quality.

All the amino-acids were vacuum-dried at 100° for at least 48 hr., weighed and made up in 0·02 M-solution. Because of the low solubility of their complexes it was necessary to use 0·001 M-concentrations of DL-norvaline and DL-norleucine. The purity of each amino-acid was assessed by two-dimensional chromatography and by potentiometric titration with 0·100 M-KOH. All samples except DL-α-alanine gave titration curves which on calculation yielded constant pK values within ±0·03 pK units over the entire curve. The pK values were calculated for each point on the curve using the method of Sörensen (1909). The values obtained were consistent with those in the literature. DL-α-Alanine of satisfactory purity was obtained after two recrystallizations from aqueous methanol.

Potentiometric titration. A Cambridge Instrument Company bench type pH meter was used with a glass electrode and saturated calomel half-cell. The titrations were carried out as outlined by Albert (1950) with minor modifications. The dissociation constant for the α-amino group was determined under the conditions subsequently used for the titrations in the presence of the metal. These titrations were made on solutions in which the ratio of acid to metal was 2:1 for bivalent and 1:1 for univalent metals. For most titrations equal volumes of stock metal and amino-acid solutions were mixed and then stirred with O_2-free N_2 for 10 min. At the midpoint of the titration the volume was 50 ml.

Calculations. The two systems to be considered in calculating the stability of each complex are:

(*a*) M^{++} + amino-acid = complex I^+ with dissociation constant = K_1,

(*b*) complex I^+ + amino-acid = complex II with dissociation constant = K_2,

where M^{++} is a bivalent metal. The overall stability of the final complex log K_s is given by

$$\log K_s = \log K_1 + \log K_2.$$

The calculation of log K_s takes account of the proportion of amino-acid in the form available for chelation at a particular pH. This form of the amino-acid is that in which the carboxyl group is ionized and the amino group unionized. All values for log K_s were calculated from the point at which one molecule of the amino-acid was bound to the metal ion. At this point the following equation was used: $\log K_s = -2 \log [Sc]$, where [Sc] is the concentration of free amino-acid available for chelation at this pH (Albert, 1950). The more critical method of calculating values for either log K_1 or log K_2 for each point on the titration curve was also used. These methods yielded values for log K_s agreeing within 0·1 unit for the amino-acids and 0·2 unit for glycylglycine.

RESULTS

Table 1 shows the log K_s values obtained for glycine and those of its derivatives in which the hydrogen atoms of the amino group were substituted. There are no values for Mg, Ca, Sr and Ba as these were too low to obtain by the present method. Fig. 1 shows the relative stability constants for glycine with Group II*b* metals. Table 2 is a comparison of the stability constants obtained by different authors using mainly potentiometric methods.

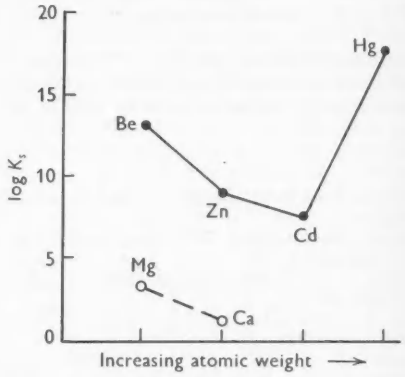

Fig. 1. Typical distribution of stability constants of Group II metals with an amino-acid (in this case glycine). The figures for magnesium and calcium are quoted from Monk (1951*c*).

DISCUSSION

Potentiometric titrations of α-amino-acids with metals of Group II showed that the two sub-groups behaved differently. The Group II*a* metals, barium, strontium and calcium either did not form complexes, or only did so with very low stability constants. In contrast, the Group II*b* metals, zinc, cadmium and mercury formed complexes with stability constants which could be readily measured. The other elements of the group behaved in different ways. Beryllium appeared to form stable complexes and was therefore associated with Group II*b*. Magnesium, however, formed complexes with low stabilities which could not be measured accurately and it behaved as though it were intermediate in properties between the two groups. Monk (1951*c*) quoted log K_s values for calcium which were well beyond the limit measurable by the method used. He also showed that magnesium had log K_s values of approximately 4·0. This is in agreement with the values of Albert (1950) and those found in the present investigation.

Throughout this investigation it was apparent that the metal was the dominating factor in determining the stability constants of the complexes. This confirmed the observation of Albert (1950) on a wide range of metals. Fig. 1 showing the relative stabilities of the metal complexes with glycine was typical of all the amino-acids studied. The order of stability was Hg > Be > Zn > Cd. So far no metal has been quoted in the literature as showing greater complex stability than mercury. The anomalous increase in stability when passing from cadmium to mercury was in accord with the work of Bjerrum (1941) on metal ammine formation. Experiments using mercuric chloride gave fallacious low results due to the mainly covalent nature of this salt. The ionized mercuric nitrate, however, gave the high values recorded here. The values for beryllium must be treated with caution as both its salts and complexes hydrolyse above pH 6. This made it impossible to assess complex formation on the alkaline side of this pH.

The effect of ligand structure on complex stability was examined. No appreciable effect was found when the hydrogen atom of the glycine amino group was substituted as in sarcosine, proline and hydroxyproline. The values in Table 1 show differences which are not temperature effects, but are slight compared with the metal effect. The substitution of the α-amino hydrogen atom by another amino-acid as in glycylglycine, however, produced a decrease in stability comparable to the metal effect.

In Table 2 a comparison is made between the results of a number of workers. In general, good agreement was obtained for glycine and α-alanine. The purpose of this part of the work was to check the results from glass-electrode measurements with those of other authors and with the solubility measurements of Monk (1951*c*). Monk (1951*a*)

Table 1. *Stability constants for metal complexes of α-amino-acids with only two ionizing groups*

(The effects of substituting the hydrogen atoms of the amino group and of carbon chain length and isomerism are shown.)

Amino-acid	Temperature (°)	pK	log K_s Be	Zn	Cd	Hg
Glycine	22·0	9·73	13·3	9·2	7·9	18·2
Glycylglycine	21·0	8·21	9·8	6·4	5·4	12·4
Sarcosine	20·0	10·22	13·9	8·8	7·5	18·7
L-Proline	17·0	10·46	14·2	9·9	8·0	20·5
L-Hydroxyproline	17·0	9·70	12·7	9·6	8·2	17·7
DL-α-Alanine	21·0	9·90	13·1	9·1	7·6	18·4
DL-α-Amino-*n*-butyric acid	20·0	9·62	12·9	8·3	6·8	18·5
DL-Norvaline	20·0	9·87	12·6	8·1	6·6	17·7
DL-Norleucine	20·0	9·92	12·8	8·5	6·9	17·8
DL-α-Amino*iso*butyric acid	19·0	10·24	12·4	8·8	7·2	18·3
DL-Valine (MBT)*	19·5	9·59	12·4	8·2	6·7	†
DL-Isoleucine	20·0	9·86	12·6	8·2	6·6	17·6
DL-Leucine	20·0	9·92	13·2	9·1	7·8	17·5

* Microbiologically tested. † Insoluble complex.

Table 2. *Comparison of stability constants in the literature of complexes formed with glycine, α-alanine and glycylglycine with various metals*

Amino-acid or peptide		Metal Cu	Ni	Zn	Pb	Co	Mg	Ca	Ag†	Cd	Hg
Glycine	(1)	15·4	11·0	9·3	—	8·9	4·0	—	—	8·1	—
	(2)	15·6*	11·1	10·0	8·9	9·3	3·4†	1·4†	3·5*	—	—
	(3)	—	11·0	9·2	9·3	8·8	—	—	3·7	7·9	18·2
	(4)	15·2	10·6	8·9	—	8·4	—	—	3·7	7·1	19·2
	(5)	15·4	—	9·7	—	8·9	—	—	—	—	—
α-Alanine	(1)	15·1	—	—	—	8·4	—	—	—	—	—
	(2)	15·4*	10·7	9·5	8·2	8·5	2·0†	1·2†	3·6*	—	—
	(3)	—	—	9·4	—	—	—	—	—	7·2	—
Glycylglycine	(2)	11·7*	7·9	6·6	6·0	5·9	1·1†	1·2†	2·7*	—	—
	(3)	—	7·6	6·4	5·8	5·8	—	—	3·1	5·4	12·4

(1) Albert (1950). (2) Monk (1951*a*, *b*, *c*). (3) Present work. (4) Flood & Loras (1945). (5) Maley & Mellor (1950).

* Solubility studies. † First constant only.

had already shown that solubility and titration measurement gave comparable results.

The results for glycylglycine agreed well with those of Monk (1951*c*). They were all measured at pH more acid than 8·0. At more alkaline pH values Dobbie, Kermack & Lees (1951) have shown that with cupric ions the peptide nitrogen also takes part in chelation. Evidence of this further chelation was obtained with mercury in this study. The titration curve obtained was symmetrical and yielded satisfactory results for log K_1 and log K_2.

The marked decrease in stabilities from glycine to glycylglycine is of interest, as according to Albert (1950) the metal bridges the carboxyl and amino groups of glycine. In the case of glycylglycine such ring formation is unlikely in view of the size of such a ring. As, however, there is evidence for the peptide losing a second proton per molecule (Dobbie *et al.* 1951) it is more probable that the metal chelates first with the terminal amino groups and then with the peptide nitrogen. Further work on metal peptide complexes is in progress.

The effect of lengthening the chain length on stability constants was small, but produced a still detectable decrease in stability from glycine to norvaline (Table 1). Norleucine, however, gave an unexpected increase in stability. The effect of isomerism in the chain was also studied. From Table 1 it will be seen that the isomers of norleucine gave different stability constants but the isomers of norvaline gave identical values.

SUMMARY

1. The stability constants of some complexes of α-amino-acids with metals of Group II of the periodic classification were measured by a potentiometric method.

2. The predominant part played by the metal in determining the stability constant was confirmed.

3. Metals of Group II*a* formed complexes with stability constants too low to be measured. Group II*b* metal complexes yielded measurable constants in the order of magnitude Hg > Be > Zn > Cd. The results of a number of investigators using a range of metals with glycine, α-alanine and glycylglycine were compared.

4. The effect of substituting the hydrogen atoms of the amino group of glycine on stability constants was studied. The only appreciable structural effect observed was the decrease in stability produced by peptide formation. Increase of chain length resulted in decreased stabilities.

I wish to thank Dr N. H. Martin for his advice and encouragement during the course of this work.

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The Crystallization of Fumarase

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Fumarase, the enzyme catalysing the reversible hydration of fumaric acid to L-malic acid, was discovered in 1911 by Batelli & Stern. Since then it has been shown to occur in a large number of plant and animal tissues, and in lower organisms.

In 1941 Laki & Laki claimed to have isolated crystalline fumarase from pig-heart muscle, but Scott (1948), Scott & Powell (1948) and Racker (1950) have shown this preparation to contain considerable quantities of aconitase and lactic dehydrogenase. Scott (1948) has also obtained amorphous fractions with over three times the specific activity of the preparation of Laki & Laki. In this communication is reported the isolation from pig-heart muscle of crystalline fumarase, homogeneous by sedimentation and electrophoretic criteria, which has a specific activity almost ten times that reported by Laki & Laki. A preliminary account of this work has been published (Massey, 1951).

METHODS

Enzyme activity determinations. Fumarase activity was determined by the spectrophotometric method of Racker (1950). The reaction was measured in the presence of 0·017 M-sodium fumarate and 0·033 M-phosphate buffer, pH 7·3, using a Beckman model DU spectrophotometer. One unit of activity was chosen arbitrarily as the amount of enzyme required to reduce the optical density of fumaric acid at 3000 A. by 0·01/min. at 20° and pH 7·3. The initial rate of reaction was calculated by plotting a graph of optical density against time of reaction.

Protein concentration. This was measured by Kjeldahl nitrogen estimations on dialysed extracts. In all calculations the protein concentration was assumed to be 6·25 times the nitrogen content. Approximate concentrations were also obtained by optical density determinations at 2770A. The optical density of 1 ml. of protein solution in a final volume of 3 ml., determined through a quartz cell of 1 cm. thickness was referred to as density/ml. (d/ml.).

Sedimentation and diffusion. The sedimentation constant was determined using a Svedberg oil-turbine ultracentrifuge and the diffusion constant using a Gouy diffusiometer (see Addendum).

Electrophoresis. Electrophoretic mobilities were determined with a Perkin Elmer electrophoresis apparatus at 1°. The current passed was 8 ma.

EXPERIMENTAL

Method of isolation

Fresh pigs' hearts were cleaned of fat and connective tissue, minced, and washed with large quantities of tap water until the washings were almost colourless. The washed mince was squeezed in cheesecloth, and homogenized with three times its weight of cold 0·01 M-Na_2HPO_4 for 3 min. The homogenate was spun for 30 min. at 1800 g in an International Serum Centrifuge, and the supernatant poured from the residue. The pH of the supernatant was adjusted to 5·2 with 1 M-acetate buffer, and the inactive precipitate centrifuged off. The clear, slightly reddish supernatant contained fumarase in almost the same quantities as the supernatant before adjusting the pH to 5·2, but the protein concentration was reduced to 10–14% of the initial value. The fumarase was then adsorbed quantitatively on $Ca_3(PO_4)_2$ gel. The amount necessary varies from preparation to preparation, and must be determined by pilot lots for maximum yield of enzyme. It is generally in the range of 4–7 ml. of gel (containing 30 mg. dry wt./ml.) for 100 ml. of solution. The $Ca_3(PO_4)_2$ was centrifuged and the supernatant discarded. The enzyme was next eluted from the gel with cold 0·1 M-phosphate buffer, pH 7·3, containing 50 g. $(NH_4)_2SO_4$/l. The fumarase can be eluted almost quantitatively from the gel—three to four elutions are sufficient to remove the bulk of the enzyme. The combined eluates were then fractionated between 45 and 60% saturation with $(NH_4)_2SO_4$, the 45–60% fraction containing the enzyme. This fraction was dissolved in distilled water, and dialysed overnight against distilled water. Considerable purification was then achieved by the addition of small amounts of $Ca_3(PO_4)_2$ to this concentrated protein solution. Sufficient $Ca_3(PO_4)_2$ was added to combine with impurities, but not sufficient to adsorb fumarase. The quantities vary from one preparation to another, and are best determined by the addition of gradually increasing amounts of gel, followed each time by centrifugation and activity and protein determinations. This was followed by another $(NH_4)_2SO_4$ fractionation, the precipitate formed on the addition of $(NH_4)_2SO_4$ to 60% saturation was centrifuged at high speed and dissolved in the minimum amount of 15% saturated $(NH_4)_2SO_4$ solution. Saturated $(NH_4)_2SO_4$ was added drop by drop until a faint turbidity was formed. The solutions of $(NH_4)_2SO_4$, which were of A.R. grade, had not been neutralized. The pH of the mixture before crystallization was between 6·1 and 6·4. After two days in the refrigerator, crystals formed in large amounts (Fig. 1). These are relatively insoluble in water; they can be washed quickly and centrifuged without much loss through solution.

Recrystallization. Fumarase crystals were centrifuged, washed with cold distilled water, centrifuged again, and dissolved in 0·01 M-phosphate buffer, pH 7·3. From this, the enzyme was precipitated by 60% saturation with $(NH_4)_2SO_4$, dissolved in 15% saturated $(NH_4)_2SO_4$, and saturated $(NH_4)_2SO_4$ added to produce turbidity as in the original crystallization. Crystallization can be greatly accelerated by seeding from a previous preparation. After two recrystallizations the preparation reaches a constant specific activity, which is from 2000 to 3000 times that of the original extract. With care, the overall yield can be 40–50%. The activity and purity data for a representative preparation are shown in Table 1.

Recrystallization from phosphate buffer, pH 6·3. Fumarase can also be obtained in crystalline form by dialysis against 0·1 M-phosphate buffer, pH 6·3. Fumarase crystals from $(NH_4)_2SO_4$ were dissolved in 0·01 M-phosphate buffer, pH 7·3, and the enzyme precipitated in amorphous form by 60% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in 0·1 M-phosphate, pH 7·3, and the pH gradually lowered by dialysis against 0·1 M-phosphate, pH 6·3.

The enzyme crystallized in the form of thin rectangular plates (Fig. 2). These crystals are almost insoluble in water and dilute phosphate solutions, but can be dissolved in dilute NaOH solution. If this is done at 0° and the pH adjusted to neutrality as soon as the crystals are dissolved, there is no loss in activity. The solution so obtained has the same specific activity as solutions of fumarase obtained from $(NH_4)_2SO_4$. All the other results reported in this paper however have been obtained with the crystals from $(NH_4)_2SO_4$.

Pig-heart protein A. From the mother liquor of the crystallization of fumarase, another crystalline protein can be obtained on the addition of a little more $(NH_4)_2SO_4$. Here crystallization proceeds best at 20–25°. The crystals, unlike those of fumarase, are very soluble in water, and appear as long thin needles without sharply defined edges. Attempts to identify the crystals have not yet been successful.

Properties of fumarase

Like other crystalline enzymes, fumarase is protein in nature; it gives a strong biuret reaction, is denatured by heating, and by extremes of acidity and alkalinity. It is reasonably stable over the pH range 5–9. The crystals are faintly birefringent in polarized light, and do not contain any ammonium sulphate. They are slightly soluble in distilled water, but readily soluble in 0·01 M-phosphate

Table 1. *Details of a representative preparation of fumarase*

Treatment	Activity (units/ml.)	Protein concn. (d/ml.)*	Purity (units/d)	Volume (ml.)	Total units	Times purified	Yield (%)
Pigs' hearts homogenized with 0·01 M-Na_2HPO_4. Supernatant	9	6·53	1·37	4800	43000	—	—
Adjusted to pH 5·2. Supernatant	8·5	0·45	19	4750	40050	14	94
Added 185 ml. $Ca_3(PO_4)_2$ gel (30 mg./ml.). Supernatant	0	—	—	—	—	—	—
Eluted four times with 0·1 M-phosphate, pH 7·3, containing 50 g. $(NH_4)_2SO_4$/l.	66	1·35	49	590	39000	36	91
Fractionated between 45–60% saturation with $(NH_4)_2SO_4$	1050	5·85	180	30	31500	132	73
Dialysed overnight against distilled water	750	3·90	192	42	31500	140	73
Added $Ca_3(PO_4)_2$ gel. Supernatant from							
0·5 ml.	750	3·70	203	42	31500	148	73
1·0 ml.	750	3·15	238	42	31500	174	73
2·5 ml.	700	2·45	286	43	30000	209	70
5·0 ml.	679	1·85	362	45	30000	265	70
10·0 ml.	510	0·85	600	50	25500	438	59
Added $(NH_4)_2SO_4$ to 60% saturation, precipitate dissolved in 15% sat. $(NH_4)_2SO_4$. Crystallized	—	—	—	—	—	—	—
Crystals dissolved in 0·01 M-phosphate buffer, pH 7·3	1200	0·48	2500	15·1	18100	1830	42
1st Recrystallization	1800	0·62	2900	9·5	17100	2110	40
2nd Recrystallization	1560	0·54	2900	10·5	16400	2110	38

* For definition of d/ml. see Methods.

buffer, pH 7·3. At pH 7·3 and 20°, in the presence of 0·033 M-phosphate, 1 g. of crystals is capable of converting 0·55 mole (i.e. 64 g.) of fumaric acid to L-malic acid/min. On the basis of a molecular weight of about 200000, as indicated by sedimentation and diffusion, this represents a turnover number of over 100000 moles substrate/min./mole fumarase, under the above conditions.

At pH 6·7 and 39° in the presence of 0·1 M-phosphate, the conditions which were used by Laki & Laki (1941), the activity was 1·30 moles of fumaric acid converted to L-malic acid/min./g. enzyme. The activity reported by Laki & Laki (1941) was 0·14 mole/min./g. enzyme.

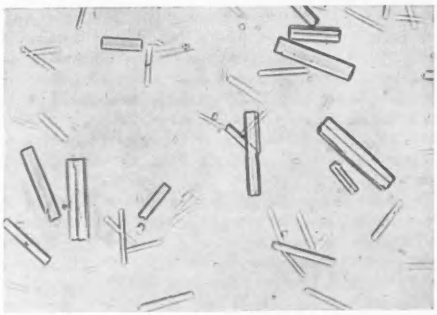


Fig. 1. Fumarase crystals from ammonium sulphate. ×600.

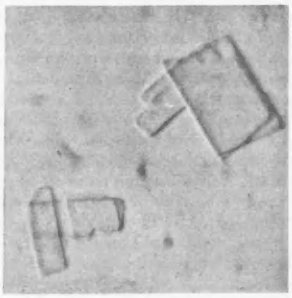
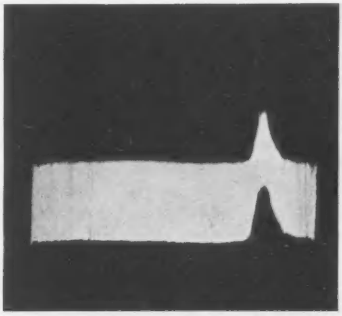


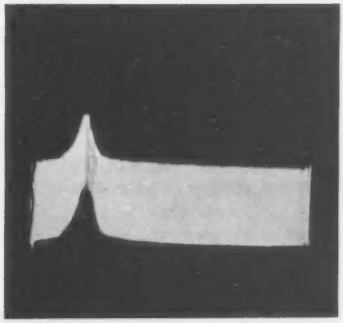
Fig. 2. Fumarase crystals from 0·1 M-phosphate, pH 6·3. ×950.

As indicated by previous studies with partially purified preparations, crystalline fumarase has a very high specificity, attacking only fumaric and L-malic acids. Maleic, D-malic, *trans*- and *cis*-aconitic, tartaric, aspartic and crotonic acids are completely unattacked by even large concentrations of fumarase.

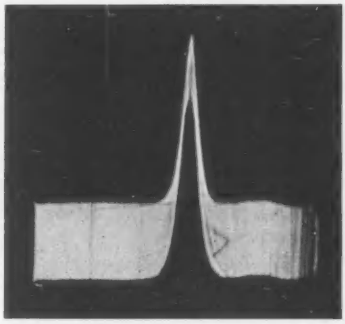
Molecular weight. The molecular weight was determined by sedimentation and diffusion measurements of a 0·7% (w/v) solution of fumarase in 0·087 M-phosphate buffer of pH 7·34. The results, which are given in the Addendum, show a molecular weight of 204 000 under these conditions, assuming a partial specific volume of 0·75.



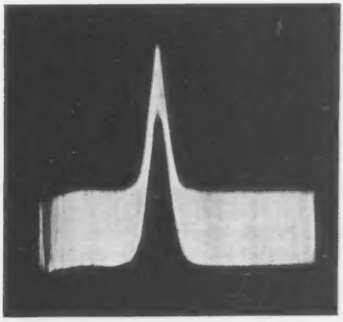
(*a*) pH 5·8. Time after beginning of exp. 5520 sec. Ascending boundary.



(*b*) pH 5·8. Time, 5520 sec. Descending boundary.



(*c*) pH 7·34. Time, 8360 sec. Ascending boundary.



(*d*) pH 8·1. Time, 7875 sec. Descending boundary.

Fig. 3. Electrophoresis schlieren diagrams.

Electrophoretic behaviour. Electrophoresis was performed at pH 8·1, 7·34 and 5·8, at a constant ionic strength of 0·21. At these pH values the material appeared to be perfectly homogeneous. The results are shown in Fig. 3 and Table 2. The slight bumps, away from the main peaks, in Fig. 3 (*c*) and (*d*) show the initial position of the boundary and are the usual boundary anomalies found with high protein concentrations.

Table 2. *Electrophoretic mobilities of fumarase*

pH	Mobility (cm.^{2} sec.$^{-1}$ V.$^{-1}$ × 10^{-5})
5·8	0·93
7·34	2·25
8·1	2·45

From the mobilities at these pH values, an extrapolated value for the isoelectric point of fumarase is between pH 5·0 and 5·4. Unfortunately, the electrophoretic behaviour of fumarase at pH values lower than 5·8 could not be studied, because of the low solubility in this pH region. However, the extrapolated value agrees well with the isoionic point of pH 5·0 as determined by exhaustive dialysis of a salt-free solution of fumarase.

SUMMARY

1. The isolation of crystalline fumarase and another, as yet unidentified, crystalline protein from pig heart is described.

2. Sedimentation and diffusion constants indicate that fumarase is a homogeneous protein of molecular weight about 200 000.

3. Electrophoresis studies at three pH values indicate a homogeneous protein. The results give an isoelectric point for fumarase between pH values of 5·0 and 5·4.

4. The turnover number of fumarase is over 100000 at 20° and pH 7·3 in the presence of 0·033 M-phosphate.

The principle of the initial stages in the purification was based on results obtained in an advanced class experiment under the direction of Dr M. Dixon, F.R.S., Dr S. J. Bach and Dr E. C. Webb at the Biochemistry Department, Cambridge. I am indebted to Dr R. Cecil and Dr A. G. Ogston of the Biochemistry Department, Oxford, for the determination of sedimentation and diffusion constants, and to Dr B. Conway and Mr D. W. F. James of the Chester Beatty Research Institute, London, for electrophoretic mobility measurements. I wish to thank Dr M. Dixon for suggesting the problem and for his advice and help in this work. The work was carried out with a grant from the Commonwealth Scientific and Industrial Research Organization, Australia.

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ADDENDUM

Sedimentation and Diffusion

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A solution of fumarase containing 0·7 g./100 ml., dialysed against a buffer containing 0·067 M-Na_2HPO_4 and 0·017 M-KH_2PO_4, was examined in the Svedberg oil-turbine ultracentrifuge by the method of Cecil & Ogston (1948) and in the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948).

A symmetrical boundary curve was obtained in the ultracentrifuge, whose area represented 96 % of the refractive increment of the solution, measured against diffusate. The sedimentation constant, corrected to its value in water at 20°, was $8{\cdot}51 \times 10^{-13}$.

The mean diffusion constant, corrected to its value in water at 20°, was $4{\cdot}45 \times 10^{-7}$. Analysis of the Gouy interference pattern (Ogston, 1949) showed that the material was not homogeneous, but contained 7 % of rapidly diffusing material; the diffusion constant of the main component was $4{\cdot}05 \times 10^{-7}$. The proportions of the main component estimated by the ultracentrifuge (96 %) and by diffusion (93 %) agree within the accuracy of these estimates. The presence of some rapidly diffusing material might have been due to incomplete dialysis.

Using the corrected value for the diffusion constant of the main component and assuming a value of 0·75 for the partial specific volume, a value of 204000 is obtained for the molecular weight. Although this is derived from quantities measured at a single concentration, it is not likely to differ much from the true value.

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The Bound Nucleotide of the Isolated Myofibril

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(*Received* 22 *November* 1951)

Since Straub & Feuer (1950) reported the presence of adenosinetriphosphate (ATP) in globular actin (G-actin) the nucleotide content of this protein has been the subject of considerable investigation. The original claim that ATP was split during the transformation of G-actin to fibrous actin (F-actin) has been substantiated by the work of Laki & Clark (1951) and Szent-Gyorgyi (1951), but the work of these authors does not support the view that depolymerization is accompanied by resynthesis of ATP. On the other hand, Dubuisson & Mathieu (1950), whilst confirming that ATP is present in G-actin preparations, could not detect any change in phosphate distribution or any change in free phosphate during polymerization. Although the above investigators do not agree as to the role of ATP in the polymerization of actin, all confirm that ATP is a component of normal G-actin preparations, whereas Snellman & Gelotte (1951), using chromatographic techniques, were unable to detect any ATP or adenosinediphosphate (ADP) in either G-actin or F-actin. These authors consider that the prosthetic group of actin is a dinucleotide containing adenine and a so-far unidentified base which can occur in phosphate rich and phosphate poor forms, corresponding to G-actin and F-actin respectively.

Actin is not alone in its property of binding adenine nucleotides, for Buchtal, Deutsch, Knappeis & Munch-Petersen (1949) have shown that when myosin and actomyosin are treated with ATP, some nucleotide is bound to the protein in such a way that it cannot be removed by repeated washing.

The investigations so far mentioned have been carried out on extracted purified myofibrillar proteins and the nucleotide content of the intact myofibril has received little attention. Using ultraviolet absorption methods Caspersson & Thorell (1942) claimed to have demonstrated the localization of ATP in the I band of resting muscle. It is not possible, however, to decide whether their results were due to adenine nucleotide or to the small amount of nucleic acid which occurs in the myofibril (Perry, 1952). In an earlier communication (Perry, 1951) a method employing collagenase was described for the isolation of myofibrils in comparatively small quantities. Preliminary analyses of these preparations indicated that the isolated myofibril contained small amounts of nucleic acid, phospholipid and acid-labile phosphorus. In the present paper a study of this latter phosphorus fraction is described, together with a method for isolating myofibrils in larger quantities which has considerably facilitated the investigation. The acid-labile phosphorus of myofibrils obtained from skeletal and cardiac muscle of the rabbit, and rat skeletal muscle, is rather constant in amount. In rabbit skeletal muscle it arises from ATP and ADP bound to the myofibril in a manner which renders these nucleotides inaccessible to the enzymes which normally attack them. This nucleotide fraction consists predominantly of ADP, and the quantities present are compatible with the view that it is associated with F-actin in the isolated myofibril.

METHODS

Preparation of myofibrils. Muscle from the hind legs and back of a rabbit was quickly dissected out, chilled in ice and minced. The mince was homogenized in a Waring Blendor for 2 min. with 8 vol. of 0·08M-borate buffer, pH 7·1. This and all subsequent operations were carried out at 0°. Centrifugation of the homogenate for 15 min. at 600 *g* was usually sufficient at this stage to give a compact sediment which was resuspended in the same volume of borate buffer as was used to make the original homogenate. The suspension was homogenized again for 2 min. This homogenate was centrifuged for 20 min. at 600 *g*, the turbid supernatant discarded and the lighter-coloured upper layer of the sediment carefully removed with the aid of a little borate buffer. This upper layer, which consisted mainly of myofibrils, was freed from coarser material by centrifugation for 5 min. at 300 *g*.

The myofibrils were washed free from granules (Perry, 1952) and soluble sarcoplasmic components by repeated resuspension in borate buffer followed by centrifugation for 20–30 min. at 600 *g*. To obtain a suspension of myofibrils which contained less than 2% of protein in solution, 4–5 centrifugations were necessary. These centrifugations were carried out as rapidly as possible because, after standing some hours in borate buffer, the myofibrils became increasingly difficult to centrifuge down at 600 *g*. It is desirable to keep the centrifugal field at this figure to ensure a satisfactory separation of granules and myofibrils. After an additional centrifugation at 300 *g* for 5 min. to remove any remaining large particles and myofibrils which may have denatured and clumped, the preparation was stored at 0° in the presence of a trace of toluene. The stored suspension was very viscous and usually contained 10–15 mg. protein per ml. By this method 5–10 g. of myofibrils could readily be

obtained from 200 g. of minced muscle. When examined microscopically with the aid of methylene-blue stain the preparations could be seen to be free from other components.

Isolation of nucleotide preparation. Myofibrils were washed twice by centrifugation and resuspension in distilled water to remove most of the borate buffer and the free phosphate which leaches out of these structures on standing. One volume of cold 60% (w/v) trichloroacetic acid (TCA) was well stirred into 11 vol. of the myofibril suspension. After standing for 20 min. at 0° the fibrous myofibrillar precipitate was centrifuged down and the supernatant filtered. When the clear filtrate had been extracted four times with ether to remove the bulk of the TCA, the aqueous layer was freed from ether by distillation *in vacuo* in the cold and finally freeze-dried.

The dry, white residue consisting mainly of borate, which acted as a convenient carrier for the nucleotide, was extracted three times with ether to remove the last traces of TCA, and then dried and stored in a vacuum desiccator at 0°. All stages of this preparation were carried out in the cold to avoid the breakdown of acid-labile phosphate. The efficacy of these precautions was shown by the fact that the ratios of free to acid-labile P in the original TCA extract and the final nucleotide preparation were identical.

Enzymic analyses. Myosin, freed from myokinase by repeated precipitation, was prepared by the method of Bailey (1942), and myokinase as described by Colowick & Kalckar (1943). Nucleotide preparation corresponding to 10–20 μg. of acid-labile P was incubated with the enzymes individually, and then with both together, in the presence of 0·1 M-glycine buffer, pH 9·0, and 0·005 M-$CaCl_2$; final vol. 2 ml. Incubations were carried out for 30 min. at 33°. Extra enzyme added at the end of this period induced no further splitting of phosphate, indicating that the reaction had gone to completion.

Spectrophotometric analysis for adenylic acid, ADP and ATP was carried out with the Beckman spectrophotometer by following the fall in absorption at 265 mμ. when the nucleotide was treated with (1) adenylic 5-deaminase, (2) myokinase and adenylic 5-deaminase, (3) potato pyrophosphatase and adenylic 5-deaminase. The enzyme preparations and the experimental procedures were carried out as described by Kalckar (1947).

Acid-labile P. The inorganic phosphate liberated when the TCA extract of the myofibrils, or an aqueous solution of the nucleotide preparation, were heated with N-HCl for 10 min. at 100°, was estimated by the method of Fiske & Subbarow (1925).

Table 1. *Acid-labile phosphorus content of isolated myofibrils*

Preparation	Condition	Source	Acid-labile P (μg./g. myofibril)
9/5	Myofibrils stored at 0°:		
	5 days	Rabbit skeletal	111
	26 days	Rabbit skeletal	82
5/6	Myofibrils stored at 0°:		
	1 day	Rabbit skeletal	121
	7 days	Rabbit skeletal	121
	9 days	Rabbit skeletal	100
	24 days	Rabbit skeletal	100
	Treated 2 days with 0·5 M-KCl	Rabbit skeletal	83
	Dialysed 2 days against 0·5 M-KCl	Rabbit skeletal	63
7/8	Freshly prepared	Rabbit psoas	108
3/8	Muscle kept 3 days at 0° (rigor)	Rabbit psoas	114
17/10	Freshly prepared	Rat hind leg	116
24/10	Freshly prepared	Rat hind leg	92
18/10	Muscle stored 1 day at 0° (rigor)	Rat hind leg	146
31/10	Muscle stored 1 day at 0° (rigor)	Rat hind leg	100
24/10	Freshly prepared	Rabbit heart	88
11/10	Freshly prepared	Rabbit heart	88
31/10	Muscle stored 4 days at 0° (rigor)	Rabbit heart	111

RESULTS

TCA extracts of myofibrils from fresh rat and rabbit skeletal muscle contained acid-labile P ranging in amount from 90 to 140 μg./g. of myofibril (the dry weight of the myofibril was taken as six times the nitrogen content). Values within this range were also obtained from muscle (rabbit psoas and rat hind-leg) which had been allowed to go into rigor by storing at 0°.

Continued washing with 0·08 M-borate, pH 7·1, did not remove the acid-labile P from the myofibrils; after storing the suspensions in this buffer for 3–4 weeks at 0° the amount had fallen by only 20–30%. During storage the acid-labile P remained bound to the myofibril and no significant amount could be detected in the clear supernatant obtained by centrifuging the suspension for 10 min. at 7000 *g*. Table 1 summarizes these results and also indicates that treatment with M-potassium chloride, which rendered 75–85% of the total protein of the myofibril soluble, only slightly reduced the acid-labile P level. A rather greater decrease was obtained in one experiment when a 0·5 M-potassium

chloride extract of the myofibrils was dialysed against 0·5M-potassium chloride for 2 days at 0°.

Experiments with heart muscle indicated that the acid-labile P level is similar to that obtained with skeletal myofibrils. It should be noted, however, that preparations from heart were not usually as satisfactory as those from skeletal muscle, because cardiac tissue does not break down so readily into single myofibrils and the latter are not so easily separated from granules.

Fig. 1 is the ultraviolet absorption curve given by a preparation of nucleotide from well washed skeletal muscle myofibrils. The curve shows a maximum at 259 mμ. and is in general similar to that given by the adenosine polyphosphates. For a

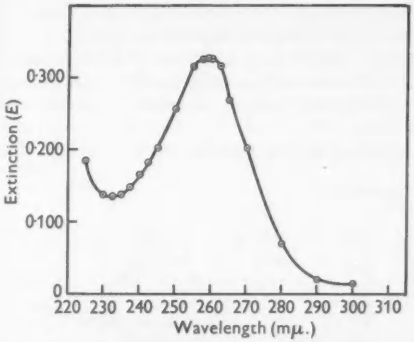

Fig. 1. Ultraviolet absorption of nucleotide from washed muscle myofibrils.

number of reasons it seemed likely that ADP or ATP could be responsible for the acid-labile P of the myofibril; to determine whether this was the case, and if so to estimate the amounts of the two polyphosphates, the nucleotide preparation was analysed by determining the amount of phosphate split from it by myosin, and then by myosin in the presence of myokinase. Table 2 shows that myosin alone split only a fraction of the acid-labile P whereas with myokinase 75–90% was liberated as inorganic phosphate. From these experiments it can be concluded that the acid-labile P arises from a mixture of ATP and ADP, with the latter making up about 80% of the whole. In no case was the total acid-labile P split by these enzymes acting in combination. This does not necessarily mean that other compounds containing acid-labile P are present, for the experience of other workers (cf. Bailey, 1949) with myosin and myokinase acting in combination indicates that it is extremely difficult to get complete hydrolysis of the acid-labile P even with the purest samples of ATP and ADP.

Potato pyrophosphatase prepared by the method of Kalckar (1947) liberated rather more than the acid-labile P from the nucleotide. On prolonged incubation in the presence of large amounts of enzyme, phosphorus was split from the nucleotide in amounts which approached the total organic P content. Under comparable conditions, strangely enough, the enzyme split only the acid-labile P of an ATP preparation, though it did slowly attack adenylic acid. Bailey (1949) and Krishnan (1949) have pointed out that there is some phosphatase (or nucleotidase) associated with the Kalckar potato pyrophosphatase preparation. If the pyrophosphatase is used in low concentration and particularly if the adenylic acid is removed as it is formed, as in the Kalckar spectrophotometric estimation of the adenine nucleotides, interference by the phosphatase activity is small.

Table 2. *Analysis of nucleotide preparation using myosin and myokinase*

(Results expressed in μg./g. of nucleotide preparation.)

Nucleotide preparation	N2	N3
Free P	503	1213
Acid-labile P	831	1395
Total organic P	—	2762
Total organic P / Acid-labile P	—	1·98
Acid-labile P calculated from amounts of ATP and ADP determined spectrophotometrically	770	1485
P liberated by myosin	107	179
P liberated by myosin and myokinase	604	1168
Acid-labile P due to ATP	214	358
Acid-labile P due to ADP	390	810
Moles ADP as % moles ADP plus ATP	79	82

Table 3. *Relative proportions of adenosine phosphates in nucleotide fraction isolated from rabbit myofibrils*

(Figures for each preparation represent the average of two or more estimations.)

Nucleotide preparation	Age of myofibril preparation (days)	(Moles % total moles adenine nucleotides) Adenylic acid	ADP	ATP
N2	10	17	75	8
N3	1	13	79	8
Average		15	77	8

In Table 3 are shown the results of enzymic analyses for the adenosine phosphates carried out by the method of Kalckar (1947). Although these estimations indicate a higher relative proportion of ADP than was obtained by assay with myosin and myokinase, the results given by the two methods are in satisfactory agreement, considering their accuracy (Table 2).

The work of Buchtal *et al.* (1949), on the adsorption of adenine nucleotides on myosin and actomyosin, suggests that during the preparation of

myofibrils, nucleotides could be picked up from solution and adsorbed on these structures in a similar way. The rather constant composition and amount of the nucleotide fraction associated with the myofibrils makes such an explanation unlikely, a view which is supported by the following experiment. ATP was added to myofibril suspensions in 0·08 M-borate buffer, pH 7·1, so that the final concentrations of acid-labile P were 10 and 100 times that bound to the myofibrils. At the higher ATP concentration the myofibrils had very obviously synaeresed, but in all cases after washing by centrifugation, and resuspension in the borate buffer, there was no significant difference between the acid-labile P content of the control myofibrils and those treated with ATP. For (*a*) control myofibrils, (*b*) myofibrils treated with ATP equivalent to 10 times, and (*c*) myofibrils treated with ATP equivalent to 100 times their acid-labile P content, the results were 127, 143 and 131 μg. of acid-labile P/g. respectively.

the myofibril. The ultraviolet absorption spectrum of the nucleotide preparation did not show any evidence of a maximum at 250 mμ., the position of maximum absorption by inosinic acid.

The fact that the acid-labile P is not split off from the myofibrils when muscle passes into rigor probably accounts, in part at least, for the residue of easily hydrolysable phosphate reported by Bendall (1951) to be present in rigor muscle.

The amounts and relative proportions of adenylic acid, ADP and ATP are similar to those which would be expected if all the nucleotides were considered to be associated with actin in the fibrous form in the intact myofibril. If actin makes up 20% of the myofibrillar protein (cf. Hasselbach & Schneider, 1951), the actin present in the myofibril would have a nucleotide content which is similar to that of the extracted F-actin analysed by Szent-Gyorgyi (1951). It is a matter of some interest to decide whether actin exists in the globular or fibrous form in the living cell. To assess the true significance

Table 4. *Nucleotide content of actin and isolated myofibrils*

(Results expressed as μmoles/g.)

Materia	Adenylic acid	ADP	ATP	Authority
Myofibrils*	0·88	2·7	0·47	—
Actin in myofibrils†	4·4	13·5	2·3	—
F-actin	—	—	8·5	Laki, Bowen & Clark (1950)
F-actin	1·6	11·7	2·4	Szent-Gyorgyi (1951)
G-actin	—	—	3·4	Laki *et al.* (1950)
G-actin	—	—	9·8	Laki *et al.* (1950)
G-actin	—	—	23	Straub & Feuer (1950)
G-actin	—	—	12–39	Dubuisson & Mathieu (1950)
G-actin	2·1	1·9	7·8	Szent-Gyorgyi (1951)

* These values are obtained by taking 5·9 as the molar ratio of ADP to ATP (average of the results obtained by the two methods described) in rabbit skeletal myofibrils containing 113 μg. of acid-labile P/g.

† Calculation made on the basis that all the nucleotide is associated with the actin which makes up 20% of the total myofibrillar proteins.

DISCUSSION

From the evidence presented it would appear that the nucleotides associated with the myofibrils are bound to its structure in such a way that although the system contains myokinase and an active adenosinetriphosphatase (Perry, 1951) they are not attacked by these enzymes; that is, so far as can be judged by the maintenance of the acid-labile P level in stored myofibrils. In this respect the bound ADP of the myofibrils showed some differences from that associated with F-actin, for Laki & Clark (1951) have reported that a short time after the addition of salt to G-actin, practically no ADP could be found. This effect they attributed to the myokinase activity of their actin preparations.

In addition to ADP and ATP, adenylic acid is also present and this compound also must be unavailable to adenylic deaminase which is present in

of the state of the nucleotide in the isolated myofibril it must be known whether the system is in the relaxed or the resting condition because it is possible, although it has yet to be proved, that contraction is accompanied by a change in the state of polymerization of actin. Electron microscope examinations (kindly carried out by Mr R. W. Horne of the Cavendish Laboratory) of myofibrils isolated by the method described in this paper show the I band to be shorter than the A band, suggesting that at some stage of the preparation a certain degree of contraction had taken place. It must be concluded then that the actin in the isolated myofibril corresponds to the contracted or partly contracted state.

In Table 4 the nucleotide analyses of actin carried out by various authors are presented. The considerable variations in these values probably reduce their reliability, and they should be compared with the rather constant values for the acid-labile P of

isolated myofibrils as shown in Table 1. Many of the actin preparations have higher ATP values than might be expected in view of the low amount of this nucleotide in the well washed muscle residue from which actin is extracted, and which must consist mainly of myofibril residues. Straub & Feuer (1950) found insignificant amounts of ATP in the acetone-dried fibre from which they extracted G-actin and concluded that ATP was produced during the water extraction of the fibre.

Assuming that the adenine content of the isolated myofibril is compatible with that found *in vivo* it is possible to speculate on the ATP distribution within the muscle cell. Normal muscle contains powerful ADP phosphorylating systems and it is likely that in the resting state all the adenine exists as ATP. This would mean that about 10% of the ATP content of the cell is associated with the myofibrils. As the latter occupy about two-thirds of the cell volume, the concentration of ATP in the extra-myofibrillar space, which is occupied by the sarcoplasm, will be 18 times that in the myofibril, assuming that the myofibrils are not interpenetrated by the sarcoplasm, or alternatively that any ATP which does penetrate into them is broken down. This concentration gradient must be an important factor affecting the accessibility of ATP to the enzyme associated with the contractile structure, the myosin adenosinetriphosphatase.

SUMMARY

1. A non-enzymic method for the isolation of myofibrils from rabbit skeletal muscle has been described.

2. Myofibrils from cardiac and skeletal muscle of the rabbit and skeletal muscle of the rat contained 90–140 μg. of acid-labile phosphorus per gram. Similar values were obtained with rigor muscle.

3. The acid-labile phosphorus of myofibrils isolated from rabbit muscle arises from adenosinediphosphate, and to a lesser extent, adenosinetriphosphate. These nucleotides are bound to the myofibril in such a way that they are not acted upon by the enzymes occurring in myofibril which normally attack them.

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Histidine α-Deaminase and the Production of Urocanic Acid in the Mammal

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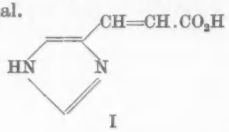
Urocanic acid (β-4(5)-glyoxalinylacrylic acid) (I), was discovered by Jaffe (1874) in the urine of one dog and was considered after its rediscovery by Siegfried (1898) to be a possible normal breakdown product of histidine. Evidence produced by Raistrick (1917, 1919) showing that it was formed from histidine by the action of certain micro-organisms, and the determination of its structure by Hunter (1912) fostered the suggestion that direct deamination played a part in the normal degradation of histidine. This point of view was supported by the later work of Kotake & Konishi (1922), Kiyokawa (1933) and Harrow & Sherwin (1926). Cox & Rose (1926), however, and Darby & Lewis

(1942), came to the conclusion that the animals, in the urine of which urocanic acid had been observed, were abnormal.



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Edlbacher and his co-workers (see review by Edlbacher, 1943) isolated from liver two enzymes which might play an important part in the breakdown of histidine. One of these, to which he gave the name histidase, degrades histidine to glutamic acid, formic acid and ammonia by fission of the iminazole ring. The other, urocanase, performs a similar function on urocanic acid. Edlbacher did not prove the existence of an enzyme capable of directly removing the amino group from histidine; and although he published a scheme showing the possibility of two distinct pathways for histidine breakdown, he considered that his evidence precluded the existence of a path requiring the intermediary production of urocanic acid.

The gravimetric methods whereby urocanic acid was first isolated were only applicable to the considerable quantities available in large volumes of urine, and could not be used easily on liver tissue or extracts. The method employed by Edlbacher & Bidder (1942), although more sensitive, could not be used to identify urocanic acid in the presence of histidine, since it employed a colour reaction characteristic of glyoxalines generally.

To determine whether histidine is at least partially converted to urocanic acid in the liver and subsequently broken down to glutamic acid by urocanase, it was essential to have available methods for identifying this possible intermediate in small quantities in the presence of histidine. The present paper records the use of two such methods. By these means, although no urocanic acid has been identified in whole liver extracts, presumably owing to the simultaneous presence of urocanase, an enzyme has been demonstrated which is capable of producing the unsaturated acid from histidine.

METHODS

Measurement of ultraviolet absorption

All the ultraviolet absorption data were obtained on a Beckman spectrophotometer, model DU. All the solutions were aqueous, and where values are given indicating differences in absorption between a test solution and a control, the absorption figures for each were measured separately against water as a blank and the difference obtained by subtraction.

Paper chromatography

Paper chromatograms of pure substances and of preparations from enzyme solutions were carried out in an upward direction (Williams & Kirby, 1948) on Whatman no. 4 paper. Two solvent mixtures were employed: (*a*) butanol, 50 ml.; water, 50 ml.; glacial acetic acid, 12 ml., the alcoholic layer being used after equilibration; (*b*) a miscible mixture; butanol, 40 ml.; ethanol, 10 ml.; water, 50 ml.

The chromatograms were developed, after a run of 2–4 hr. at 30°, by spraying successively with a cold aqueous solution of diazotized *p*-chloroaniline (Edlbacher, Baur, Stachelin & Zeller, 1941) and N-NaOH. The latter spray could be replaced by suspension of the paper in an atmosphere of NH_3 vapour. The colours which varied between yellow and red persisted strongly, assuming a more intense red colour with age.

Preparation of urocanic acid

Urocanic acid was prepared by decarboxylation of a sample of 4(5)-glyoxalinylmethylenemalonic acid, which was kindly supplied by Dr A. Neuberger, F.R.S. The method of decarboxylation was essentially that of Akabori, Ose & Kaneko (1940) employing boiling pyridine. Most of this reagent was removed by distillation under reduced pressure and impure urocanic acid precipitated on pouring the residue into ether. Persistent washing with ether was necessary to ensure the removal of all the pyridine; the acid was then recrystallized from water to give material with a m.p. (uncorr.) of 226–228°. The absorption curve for this material is given in Fig. 1. Purity of the sample was determined in two ways. Freedom from pyridine was proved by the absence of the characteristic absorption peak of pyridine at 256 mμ. Freedom from malonic acid derivative was proved by partition chromatography. The R_F values of 4(5)-glyoxalinylmethylenemalonic acid and urocanic acid in butanol-acetic acid are respectively 0·30 and 0·52; thus separation is complete and small amounts of the malonic acid derivative can be detected in the presence of large quantities of urocanic acid.

A further sample of urocanic acid was obtained by an adaptation of the method of Engeland & Kutscher (1913) for the production of histidine betaine, and its subsequent breakdown to urocanic acid and trimethylamine by the method of Barger & Ewins (1911). The material prepared by this method had properties identical with those of the sample prepared from 4(5)-glyoxalinylmethylenemalonic acid.

Identification and estimation of urocanic acid

Histidine at pH 7·4 has a relatively low absorption peak at 262 mμ., and the molecular extinction coefficient of urocanic acid at the same pH is 200 times as great. Thus it is possible to use ultraviolet absorption as a method for the identification of urocanic acid in the presence of histidine.

The variation in intensity of absorption and in the position of the peak with changes in pH are such as could be expected with an amphoteric substance. The four curves in Fig. 1 demonstrate the magnitude of these pH variations. The curve at pH 4·5 represents the absorption of urocanic acid in aqueous solutions at a concentration of 4 μg./ml. The other curves are those for the same concentration of the acid brought to the stated pH by the addition of NaOH or HCl. It was found that in the presence of acid there was much irrelevant absorption with the systems under investigation and pH values around neutrality were, therefore, always employed when examining solutions for the presence

of urocanic acid. For although the peak at 278 mμ. is lower it is well defined owing to the pronounced minima at nearby wavelengths.

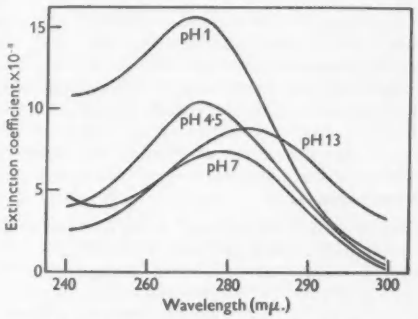


Fig. 1. Absorption curves of urocanic acid (4 μg./ml.), brought to the pH values indicated by the addition of appropriate amounts of sodium hydroxide and hydrochloric acid. The curve of pH 4·5 represents the actual curve of the acid dissolved in distilled water.

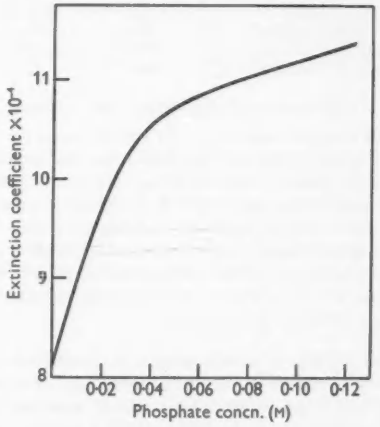


Fig. 2. The effect of phosphate ion concentration on the extinction coefficient at $\lambda_{max.}$ (278 mμ.) for solutions of urocanic acid brought to pH 7 with sodium hydroxide.

Since in many of the systems to be examined, phosphate was present, its effect on the absorption was examined. As can be seen from Fig. 2, the absorption is considerably increased by the presence of even small concentrations of phosphate. Although the rate of increase falls off above a phosphate concentration of more than 0·05 M, there is a slight but steady increase at greater concentrations. The graph shows the absorption at 278 mμ., and pH 7·6. This effect was also observed with borate; but not with other commonly occurring ions.

Solutions of urocanic acid, kept at normal temperatures in diffuse daylight, show variable changes in absorption. The value may fall by as much as 20% in 3–4 days, but the presence of phosphate appears to retard the diminution of absorption.

Owing to the variations in absorption which are mentioned in the two preceding sections, it is impossible to obtain quantitative comparisons between solutions with entirely different pre-treatment, but for comparison of two solutions which have been treated in an identical manner, the method is regarded as quantitative. Over the range of 1–9 μg./ml. in aqueous solution, the relationship between absorption and concentration of urocanic acid is linear. This covers the whole effective range of the spectrophotometer, using a 10 mm. cell. Higher concentrations can be read after dilution.

Examination of liver extracts

Choice and preparation of material. In most of the work reported here, cat-liver extracts were employed, but the technique was identical with extracts prepared from the livers of other animals. Livers were removed from cats killed by severance of the carotid artery and were washed free of superficial blood. The tissue was macerated to a fine cream in 2·5 times its weight of physiological saline. The grosser debris after 20 min. in the macerator (Townson & Mercer top-drive type) was removed by filtration after addition of kieselguhr equivalent to half the wet weight of the liver. The resultant opalescent solution was poured into 2 vol. of acetone at a temperature of less than 5°. The precipitate was filtered off at the pump. The cake, washed once with acetone, was dried at room temperature and stored at 0°. The powder was not completely soluble in water or the buffer used, but by agitation could be brought into a sufficiently fine suspension to withstand centrifuging under a force of 3500 *g*.

Properties of the extract powder. A sample of the original macerate, filtered through a 3 mm. layer of paper pulp on a sintered funnel, gave an indeterminate curve showing marked absorption in the range 240–300 mμ., but no peak at 278 mμ. After ultrafiltration through a collodion membrane most of the absorption disappeared, showing it to be due to material of large mol.wt. There was no peak in the region of that associated with urocanic acid. Similar experiments on an aqueous or phosphate buffer extract of the acetone-dried powder prepared from the macerate failed to demonstrate the presence of any material with properties similar to urocanic acid. A sample of the extract from an acetone-dried powder that had been kept for 2 months at 0°, showed a marked peak in the neighbourhood of 278 mμ. Dialysis of the sample against running water for 24 hr. completely removed the peak, showing the material responsible for the absorption to be water-soluble and of low mol.wt.

Activity of liver powder

The incubation of a fresh acetone-dried liver extract with histidine caused a marked increase in absorption with a peak at 278 mμ. Such an increase was produced by a heat-labile system, no increased absorption being observed when histidine was incubated with boiled preparations. The same histidine α-deaminase is suggested for the enzyme concerned.

Method of testing enzyme activity. Except when various concentrations of enzyme and substrate were examined for the specific purpose of determining concentration effects,

the following procedure was employed for assessing the amount of enzyme in a given preparation. Enzyme powder (50 mg.) and solid phosphate buffer mixture, pH 7·6, (50 mg.) were dissolved in 5 ml. of water. To this mixture was added 5 ml. of a solution of histidine hydrochloride in water (1 mg./ml.) brought to pH 7·6 with NaOH. Water (5 ml.) was added in the case of the blank. The preparations were incubated at 30° for 18 hr. in the presence of 0·2 ml. of toluene in 50 ml. flasks covered with a 30 ml. beaker. After incubation the flasks were placed in boiling water for 5 min. to inactivate the enzyme, coagulate the protein and remove the small quantity of toluene. The solid was then removed by centrifuging, and the volume of the supernatant made up to 10 ml. 1 ml. of this solution was diluted with water to 50 ml., to give the solution for measurement in the spectrophotometer. The curves observed on incubation of the enzyme in the presence and absence of histidine are given in Fig. 3.

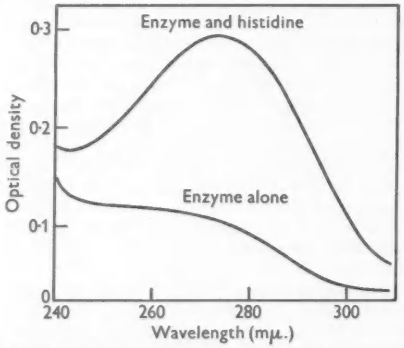


Fig. 3. Absorption curves of enzyme and enzyme-substrate mixtures after incubation at pH 7·8 for 18 hr. 50 mg. liver powder, 50 mg. phosphate buffer powder, and either 10 ml. of a 0·1% solution of histidine hydrochloride or 10 ml. of water.

Evidence for the identification of the enzyme product as urocanic acid

Examination of the material produced during the incubation of the enzyme preparation with histidine showed that changes in absorption brought about by pH were identical with those observed in pure solutions of urocanic acid. For this experiment buffer solutions or acid or alkali were used instead of water for the 50-fold dilution prior to measurement of the absorption. The changes observed are exactly as those shown in Fig. 1.

For the chromatographic identification of the material as urocanic acid all the quantities employed in the normal test were multiplied ten times. After the completion of the incubation period and after the coagulation and removal of most of the protein by filtration, the solution was concentrated to 3 ml. under reduced pressure, and the smaller molecules separated from the residual protein by ultrafiltration through a collodion membrane. A chromatogram of this material in butanol/acetic acid showed two main spots and two fainter ones. The R_F values of these and of a number of other imidazole derivatives are given in Table 1. In butanol/ethanol/water the movement of the lower two spots was only slight, and only after very long runs did separation occur. The two main spots coincide with those for histidine and urocanic acid; the other two have not yet been identified.

Table 1. *R_F values of certain glyoxaline derivatives in butanol/acetic acid/water and butanol/ethanol/water mixtures*

(The two unknown substances 1 and 2, were produced by the action of the liver preparation on histidine.)

Substance	R_F Butanol/acetic acid/water	R_F Butanol/ethanol/water
Urocanic acid	0·50	0·43
Histidine	0·11	0·10
β-Glyoxalinylpropionyl chloride	0·25	0·37
Glyoxalinylmethylene-malonic acid	0·30	—
Methylglyoxaline	0·39	0·52
Dicarboxyglyoxaline	0·0	0·09
Unknown substance 1	0·29	0·35
Unknown substance 2	0·13	0·14

Distribution of properties of the enzyme

The enzyme has been shown to be present in the livers of cats, rats, rabbits and dogs, but not in the following tissues: the pancreas, kidney, duodenum, stomach, spleen and thyroid of the pig or heart of the horse. Nor is it present in *Escherichia coli* cells, although a casual contaminant of a histidine solution, which proved to be a Gram-negative diplococcus, produced a filtrate containing urocanic acid after 3 weeks at 18°.

Table 2. *The effect of changes in concentration of enzyme and substrate on the ultraviolet absorption of histidine/histidine α-deaminase systems, after incubation at* 30° *for* 18 *hr. at* pH 7·6

(The difference in $E_{max.}$ at 278 mμ. was calculated by subtracting the curve obtained from the control enzyme preparation incubated without histidine, from that obtained after incubation in the presence of histidine. This also applies to the optical density figures in Table 3.)

Concentration of liver powder added (mg./ml.)	Difference in $E_{max.}$ Concentration of added histidine (mg./ml.) 0·1	0·2	0·5	1·0
0·5	0·007	0·010	0·018	0·028
1·0	0·009	0·013	0·023	0·039
2·0	0·019	0·026	0·038	0·056
5·0	0·027	0·051	0·084	0·114

In Table 2 are shown the results of a typical experiment in which both the concentrations of the

enzyme and of the substrate have been altered. The constants of the enzyme reaction have not yet been determined, since they will have little significance until purer preparations are obtained.

Enzyme activity was completely suppressed at pH values below 5; the pH optimum was found to be in the neighbourhood of 7·8. Citrate and phosphate buffers were employed to cover the whole pH range, and the figures for those experiments with phosphate were corrected for the enhancing effect of that ion on absorption (Table 3).

Table 3. *The effect of changes in* pH *on the ultraviolet absorption at* 278 *mμ. of histidine/histidine α-deaminase systems incubated for* 18 *hr. at* 30°. *The figures for the experiments with phosphate buffer are corrected for the enhancing effect of the phosphate ion* (*see Fig.* 2)

Buffer	pH	Differences in optical density
Citrate	3·0	0·012
	3·9	0·016
	5·1	0·003
	6·1	0·019
Phosphate	7·0	0·012
	7·2	0·008
	7·4	0·019
	7·6	0·098
	7·8	0·166
	8·0	0·144
	8·2	0·092

Old enzyme preparations which show the marked increase in urocanic acid content mentioned above are almost inactive, measured by increase of absorption on incubation with histidine. However, after dialysis and the removal of the urocanic acid the activity of the system increases to its normal value (Fig. 4).

DISCUSSION

Since it contains three conjugated double bonds, urocanic acid is expected to show considerable absorption in the ultraviolet. In fact the increase over histidine with only two double bonds is about 200-fold. This raises the molecular extinction coefficient to a level at which it is of use in the estimation of the acid in biological materials. The variability of the absorption renders the method not fully quantitative, but it is still of use in comparing similarly treated solutions. When associated with partition chromatography, ultraviolet absorption gives an admirable method of identifying the acid with a reasonable degree of certainty, but awaiting chemical characterization for absolute proof.

The reason for the decay of absorption on standing may be associated with the possibility of *trans-cis* interconversion which Edlbacher & Heitz (1943) have shown to occur quite rapidly at 100° and to be photocatalysed. Whether phosphate acts as a stabilizer of the *trans* form or enhances the absorption in some other fashion cannot yet be decided.

The absence of urocanic acid from fresh liver extracts is consistent with urocanic acid being on the pathway of histidine metabolism. Assuming that urocanase catalyses a faster reaction in the destruction of urocanic acid than histidine deaminase does in its production, not much urocanic acid would be expected to accumulate. The few

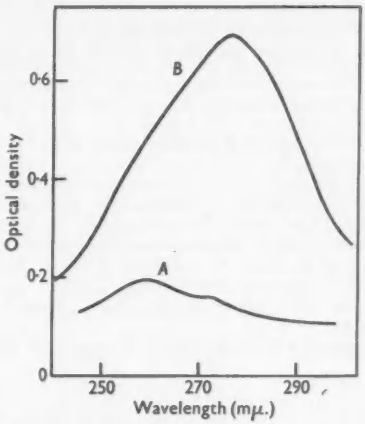
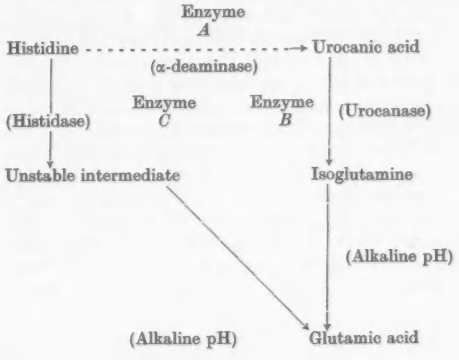


Fig. 4. Effect of dialysis on aged preparations. *A*, difference in optical density between enzyme and enzyme-substrate mixture of aged preparation before dialysis; *B*, similar curve obtained after dialysis of enzyme powder for 2 days against running water. The conditions of the experiments were in each case as given in legend to Fig. 3.

animals which have been shown to produce urocanic acid may not, therefore, have called into play a fresh metabolic pathway, but may merely have suffered the blocking of a single step in an enzymic chain. Until it is possible to measure quantitatively



the amount of urocanic acid produced it will be impossible to assess what fraction of the histidine is metabolized by the route involving urocanic acid.

The hypothetical system, shown on the previous page, proposed by Edlbacher would, however, appear to have more foundation in fact than he thought, since both enzymes *A* and *B* as well as *C* have now been shown to exist side by side in liver.

SUMMARY

1. Methods for the identification of urocanic acid (β-4(5)-glyoxalinylacrylic acid) based on its ultraviolet absorption spectrum and on its separation from other iminazole derivatives by partition chromatography are described.

2. No urocanic acid has been observed in fresh liver homogenates or extracts from acetone-dried powders obtained from aqueous extracts of liver.

3. An enzyme capable of producing urocanic acid from histidine has, however, been shown to be present in such aqueous extracts.

4. The name histidine α-deaminase is suggested for this enzyme.

5. The enzyme has an optimum pH in the region of 7·8.

6. It appears to be completely inhibited by the product of its reaction.

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Volatile Fatty Acids of *Ascaris lumbricoides* from the Pig

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During a series of investigations of the pharmacology and physiology of *Ascaris lumbricoides*, extending from 1940 to 1949, our attention was constantly drawn to the peculiar and characteristic odour emitted by these parasites and by saline media in which specimens had been kept. Bunge (1890) appears to have been the first to attribute this odour to lower, steam-volatile fatty acids, but it was left to Weinland (1901, 1904) to show that the principal acids present are a valeric and a caproic acid. Similar substances are present in another parasitic nematode, *Parascaris equorum* (Schimmelpfennig, 1903). Flury (1912), working with *A. lumbricoides*, demonstrated the presence of volatile fatty acids, especially valeric and caproic, in the tissues of this worm as well as in saline media in which it had been kept. There has, in the past, been a good deal of discussion regarding the origin of these acids, some attributing them to bacterial activity, while others maintain that they are produced by the worms themselves. The presence of relatively very high concentrations of these compounds in the body fluid and throughout the tissues would make it appear certain that the acids are produced by the worms themselves. A comprehensive review of earlier work on these acids has been given by Hobson (1948).

Although a dozen or more authors have studied the chemistry of these substances, their precise nature is still not definitely known. The results of Kruger (1936) were perhaps the most conclusive data published when our own work began, but even these are unconvincing. He collected the acids by steam-distillation of large volumes of saline in which his specimens had been accommodated. By fractional distillation of the product he obtained two main fractions, one consisting of a valeric and the other of a caproic acid, from both of which he prepared the anilides. He concluded that both acids

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are branched-chain compounds and, in the case of the valeric fraction (m.p. of anilide 98°), that it was neither the *n*-acid (m.p. of anilide 63°) nor the *iso*-acid (m.p. of anilide 112°), but must probably be the optically active methylethylacetic acid (m.p. of anilide of the racemic acid, 108°). Trimethylacetic acid was excluded on the grounds that it is solid at room temperature, while Kruger's fraction was liquid. The only confirmation in support of his identification of the optically active acid was the fact that mixtures of the anilides of the natural product and synthetic racemic acid, melted between 98 and 108°.

As we were able to obtain considerable amounts of the acids in the course of other investigations, we felt it was worth while to attempt a more positive identification. The work reported here was completed in 1949, but remained unpublished because we hoped at that time to carry it further. But in view of the recent work of Bueding & Yale (1951), it has seemed to us proper to record our observations. A brief account of this work was communicated to the Biochemical Society in London on 24 March 1950 (Baldwin & Moyle, 1950).

MATERIAL AND METHODS

Worms were obtained from the St Edmundsbury Co-operative Bacon Factory, to the manager of which we wish to express our gratitude for a regular and abundant supply of material. After washing in warm water, the specimens were conveyed to Cambridge in large thermos vessels containing the 'keeping medium' described elsewhere (Baldwin & Moyle, 1947), previously heated to 38°. On arrival in the laboratory the worms were drained, dried on filter paper, opened by a longitudinal incision and allowed to drain on a funnel, the stem of which was loosely plugged with glass wool. The clear, deep-red perienteric fluid thus collected, accounted for approximately one-third of the live weight of the worms and contained on the average about 40 m-moles of steam-volatile fatty acids per litre.

Proteins were precipitated by the addition of 0·8 vol. of 10N-H_2SO_4 and 1·0 vol. of 30% Na tungstate. The precipitate was removed in some cases by filtration and in others by centrifugation. To each 100 ml. of the clear, colourless fluid 25 g. $MgSO_4.7H_2O$ were added and allowed to dissolve. The whole was then distilled until crystallization began in the hot. The distillates were titrated with N-NaOH (phenolphthalein); the average yield was equivalent to 38·0 ml. N-NaOH/l. of perienteric fluid. A 10% excess of N-NaOH was added before evaporating the distillates *in vacuo* to about 2 ml. An excess of finely powdered $KHSO_4$ and two drops of 10N-H_2SO_4 were added, and the resulting dry powder was extracted in each case with about 50 ml. of $CHCl_3$ containing 5% (v/v) *n*-butanol in small successive portions.

Attempts were at first made to analyse the mixed acids by the distillation method of Friedemann (1938), and by fractional crystallization of the phenylhydrazides and piperazinium salts, before and after fractional distillation. The results obtained were discouraging, and we next tried the method of partition chromatography of Elsden (1946). This method, however, did not allow separation between caproic and valeric acids or between these and butyric acid. We were accordingly led to investigate the possibility of using heavily buffered silica partition columns. Details for their preparation and operation have already been described (Moyle, Baldwin & Scarisbrick, 1948), and in particular the performance of columns I–III. In Table 1 characteristics of column IV, which uses as 'buffer' KH_2PO_4 only, are given. This method was applied to our present problem, after preliminary distillation of the aqueous solution in presence of HgO (Friedemann, 1938) to remove formic acid. The results obtained are summarized in Table 2.

Table 1. *Behaviour of aliphatic acids* (C_2–C_5) *on column IV*

Butanol (%)	Eluted	By-passed	Retained
1	C_5	C_5 (part) and higher	C_2 and lower
10	C_4		
30	C_3		

RESULTS

Fraction C_1. It was found in preliminary experiments that the mixed acids, in the form of their sodium salts, decolorized iodine and bromine water, indicating the presence of small quantities of un-

Table 2. *Steam-volatile acids obtained from* A. lumbricoides

(F_1 and F_2 were duplicate analyses carried out on one sample.)

	Percentage of total steam-volatile acids					
Sample	C_8	C_6	C_5	C_4	C_3	C_2
B	1·48	40·9	40·0	1·14	4·06	12·4
C	0·78	41·2	43·5	1·72	4·73	8·23
D	29·8		50·8	2·75	4·31	12·6
E	0·37	34·1	46·2	1·89	5·12	12·6
F_1	33·8		49·4	—	3·78	—
F_2	33·7		49·3	—	3·93	—
Averages	**35·7**		**46·0**	**1·9**	**4·4**	**11·1**

saturated or reducing substances. Preliminary distillation of the acids in the presence of HgO, as recommended by Friedemann (1938) for removal of formic acid, yielded distillates which failed to react with either iodine or bromine water. It seems possible that the reducing material consisted of formic acid, small amounts of which have been found in *Ascaris* by other workers, e.g. Flury (1912). The amount present, calculated on the basis of iodine value, corresponded to only about 2 % of the total acids present, and we therefore did not concern ourselves further with this fraction.

Fractions C_2 *and* C_3. C_2 and C_3 together accounted for 15·5 % of the total acids and corresponded in their chromatographic behaviour to acetic (11·1 %) and propionic (4·4 %) acids respectively.

Fraction C_4. To obtain enough of the C_4 acid for identification we used silica partition columns 30 mm. in diameter, each containing 20 g. of silica and proportionate quantities of the other reagents. The original $CHCl_3$-butanol extract (80 ml.) was placed on a column of type II and the large C_6 and C_5 fractions eluted, the first with 1 % butanol in $CHCl_3$, and the second with 10 % butanol in $CHCl_3$. The column was then allowed to run dry, the silica removed, and, after intimate admixture with an excess of finely powdered $KHSO_4$, extracted with 100 ml. of 10 % butanol in $CHCl_3$, in successive portions. The extract was concentrated by adding a small excess of N-NaOH, evaporating to dryness, acidifying in the usual way and extracting with 20 ml. of 5 % butanol in $CHCl_3$. The whole extract was rechromatographed on a type IV column of standard size (5 g. silica) and the C_4 fraction eluted. A second 80 ml. sample of the original $CHCl_3$-butanol extract was worked up in the same way. The combined C_4 fractions were now neutralized, recovered and again chromatographed on a type IV column. Titrations were carried out in the usual manner until the C_4 acid began to be eluted and subsequent samples were collected without titration until the whole fraction had been eluted. Titration of a small sample indicated that, in all, 19 mg. of the C_4 acid had now been obtained in 60 ml. of extract. The theoretical amount of piperazine hexahydrate in ethanol was now added and the whole evaporated under reduced pressure. On addition of a few drops of acetone to the resulting oil immediate crystallization took place. After standing overnight at 3° the product was filtered off (m.p. 115–117°; mixed m.p. with the piperazinium salt of *n*-butyric acid 116–117°). The product was recrystallized from acetone (m.p. 117–118·5°; mixed m.p. with the derivative of *iso*butyric acid 65° approx.).

The C_4 acid is thus identified as *n*-butyric acid. (All m.p.'s uncorr.).

Fractions C_5 *and* C_6. Samples of the mixed acids were put on to 20 g. columns of type II. The C_6 fraction was eluted with 1 % butanol in chloroform and the C_5 acids with 10 % butanol in chloroform. Fractions obtained in this way from each of several columns were massed together and purified on 20 g. columns of type III, from which the C_6 acids were eluted with 10 % butanol in chloroform, and C_5 acids with 30 % butanol in chloroform.

After recovery of the acids in the usual way, the fractions were separately treated with the theoretical amounts of piperazine hexahydrate, in repeated attempts to obtain crystalline derivatives, but in every case oily products were obtained which failed to crystallize, even on prolonged standing. Since the piperazinium derivatives of all four isomers of valeric acid and of *n*- and *iso*-caproic (hexanoic) acid were readily obtained individually from pure samples of each, we concluded that the natural C_5 and C_6 fractions consisted of mixtures containing more than one isomer in each case. Further chromatographic experiments were therefore made in the hope of resolving the mixtures.

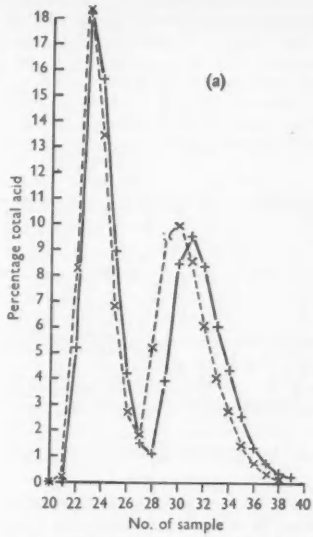
Chromatographic examination of C_5 *fraction*

Partition analysis. The behaviour of the C_5 fraction on our usual buffered columns (type III) is depicted in Fig. 1, which also shows the results of running a series of mixed chromatograms with known acids. As previously shown (Moyle *et al.* 1948), *n*-valeric and trimethylacetic acids are separable from *iso*valeric and methylethylacetic acids, but neither pair can be resolved by this method. The C_5 fraction from *A. lumbricoides* behaves in the same way as *iso*valeric and methylethylacetic acids, whether separately or in mixtures, and comes through significantly later than *n*-valeric and trimethylacetic acids. This led us to the conclusion that the C_5 fraction must include one or more of three compounds, namely, *iso*valeric, (+)- and (−)-methylethylacetic acids.

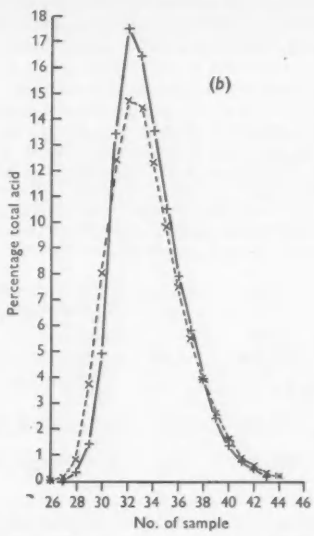
Adsorption analysis. Following the indications given by Claesson (1946), we carried out experiments on short adsorption columns of silica, previously dried at 800°, using pure specimens of the valeric acid isomers dissolved in *cyclo*hexane. In this way it was found that *n*-valeric and trimethylacetic acids could be separately estimated by frontal analysis; mixtures of either, with other isomers or with the worm acid(s), could not be analysed. Mixtures of *iso*valeric and methylethylacetic acids showed no signs of separation.

Although this part of our work failed to carry us any further, it is mentioned here since *n*-valeric and trimethylacetic acids are inseparable by our partition columns. Their separation and estimation by frontal analysis on adsorption columns thus forms a useful supplement to the partition technique.

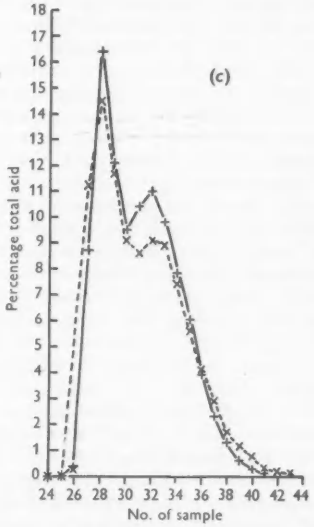
Further examination of C_5 *and* C_6 *fraction.* Having failed so far in our attempts to resolve either the C_5 or C_6 fractions into their isomeric components by chromatographic methods, we now reverted to more classical techniques involving the use of larger quantities of material. The acids used in this part of the work were obtained by steam distillation of large, bulk samples of intact worms previously submitted to digestion by 30 % (w/v) sodium



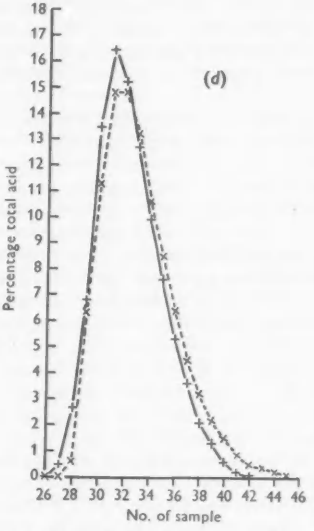
×---×, Trimethylacetic + methylethylacetic acid; +—+, trimethylacetic + *iso*valeric acid.



×---×, Worm acid; +—+, worm + methylethylacetic acid.



×---×, *iso*- + *n*-Valeric acid; +—+, worm + *n*-valeric acid.



×---×, *iso*Valeric acid; +—+, worm + *iso*valeric acid.

Fig. 1. Chromatographic behaviour of C_5 worm acid and isomers of valeric acid; mixed chromatograms.

hydroxide, first for several weeks at room temperature and then on the boiling-water bath, and subsequently acidified with sulphuric acid.

Approximately 9 g. of the mixed worm acids were refluxed for 5 hr. with 40 ml. dry methanol containing 0·5 ml. conc. H_2SO_4. Methanol (30 ml.) was removed by distillation and the residual mixture of methyl esters extracted with ether. The ethereal layer was washed five times with 2% aqueous K_2CO_3, dried over K_2CO_3 and distilled to remove ether. The mixed esters were now fractionally distilled from a small flask of about 25 ml. capacity. The following fractions were collected:

Fraction	B.p. (°)	Weight (g.)	Probable composition
1	34–36	—	Ether
2	36–100	0·86	Methyl esters of acetic, propionic and butyric acids
3	100–120	2·02	Methyl esters of valeric acids
4	120–132	2·43	Methyl esters of caproic acids

Since fraction 4 contained two main sub-fractions, boiling at 129° and 131–132°, it seems probable that two isomeric forms of caproic acid are present. These cannot be either *n*-caproic (b.p. of methyl ester 150°) or methylethylpropionic acid (b.p. of methyl ester 141°) and must therefore be other branched-chain caproic acids. The fraction was found to be optically active ($[\alpha]_D = -2·245°$) and must therefore contain at least one optically active acid.

The main part of fraction 3 came over at 115–117° and might consist of the methyl esters of *iso*valeric (b.p. 116°) and (+)- and (−)-methylethylacetic acids (b.p. 113–115°). This fraction, too, proved to be optically active ($[\alpha]_D = -1·405°$) and must, therefore, contain at least some methylethylacetic acid. Because their boiling points lie so close together, there seemed little prospect of analysing this mixture of esters by fractional distillation, and still less of analysing the corresponding mixture of acids ((±)-methylethylacetic acid, b.p. 173°; *iso*valeric acid, b.p. 174°). As our supplies of the acids were now running low we made no further attempts to fractionate the C_5 acids by distillation methods, but turned instead to infrared spectroscopy. The C_5 fraction of the worm acids (1–2 g.) was obtained in the pure state by repeated chromatography as for the C_4 fraction, described above. The acids were titrated with N-sodium hydroxide, the sodium salts thus obtained distilled to dryness, and dried *in vacuo* over phosphorus pentoxide. A pure, dry sample of the free acids was obtained by intimately mixing the dry sodium salts with finely powdered dry potassium hydrogen sulphate and distilling the acids in a flask similar to that used by Ellis (1934).

In order to determine whether the C_5 acid obtained above was methylethylacetic acid or *iso*valeric acid, the infrared spectra of pure samples of these acids and of the unknown were determined. The spectrometer used was a Perkin Elmer Type 12B, and the spectra were obtained between 1800 and 700 cm.$^{-1}$ using a rocksalt prism. The liquid samples were approximately 0·005 mm. in thickness. The absorption curves obtained are shown in Fig. 2.

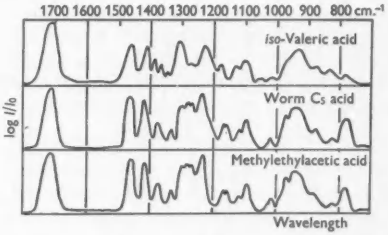


Fig. 2. Infrared absorption spectra of C_5 acids.

Although the two pure acids have a number of absorptions in common, there are considerable differences in their spectra, notably in the region 1550–1150 cm.$^{-1}$. In particular, *iso*valeric acid has strong absorption peaks close to 1300 and 1220 cm.$^{-1}$, whereas methylethylacetic acid has a marked absorption distinct from those of *iso*valeric at about 1230 cm.$^{-1}$. It can be seen immediately from the figure that the spectrum of the unknown material very strongly resembles that of methylethylacetic acid throughout the region investigated, and differs in many respects from that of *iso*valeric acid. The only detailed difference between the spectrum of the unknown acid and of pure methylethylacetic acid is an additional faint absorption in the former, which shows as a shoulder in the spectrum near 1210 cm.$^{-1}$. This presumably corresponds to a small amount of impurity, which might possibly consist of a little *iso*valeric acid. It is clear, however, that the unknown acid is very nearly pure methylethylacetic acid, with an impurity content of probably less than 10%.

It is not possible, of course, to distinguish by spectroscopic means between the (+)- and (−)-forms of this acid. The polarimetric data already mentioned indicate that, in the acid as analysed, there was a small preponderance (about 6%) of the (−)-isomer. There remained, however, the possibility that extensive racemization might have taken place in the course of preparation of these particular batches of acids. The most likely step at which racemization might have occurred was during the digestion of the worms with 30% sodium hydroxide for some weeks in the cold, followed by several hours in the hot. This possibility was investigated in the following manner.

Approximately 50 g. of synthetic (±)-methylethylacetic acid was partially resolved with brucine, according to the procedure used by Schutz & Marckwald (1896). The product, after recovery, showed $[\alpha]_D = -9{\cdot}6°$, indicating the presence of the (+) and (−) acids in the proportion of 20 and 80% approximately. This material was heated on a boiling-water bath for 5 hr. with 30% NaOH and then allowed to stand at room temperature for some weeks. Despite this treatment there were no signs of racemization, and we therefore think that the optical properties of the products of fractional distillation of the methyl esters of the worm acids, correspond to those of the natural material.

Accordingly, we have to conclude that at least 90% of the C_5 fraction consists of optically active methylethylacetic acid, with the (−)-form slightly preponderating over the (+)-acid.

Fraction C_6. As has been pointed out, we were unable to resolve the C_6 acids whether by chromatography, fractional crystallization of the piperazinium salts, or fractional distillation of the methyl esters. The possibilities of achieving separation of the acids themselves by fractional distillation seemed remote and the only promising line for further approach appeared to lie in infrared spectroscopy. This method, however, could not be applied, since the necessary reference substances were not available to us. The nature of the C_6 acid(s) therefore remains unknown for the present.

Fraction C_8. This fraction consisted of a mixture of acids containing more than six carbon atoms, and, since it accounted for only 1% of the total acids, was not examined further.

DISCUSSION

The presence of formic, acetic, propionic and *n*-butyric acids in a wide range of biological materials is well known, and they are, moreover, among the principal products of the digestion of cellulose by the rumen contents of the sheep and other herbivores (McAnally & Phillipson, 1944). That these acids may also be formed by bacterial activity in the alimentary tract of the pig is possible, and the fact that they occur in laboratory media in which specimens of *Ascaris* have been incubated has led many workers to the conclusion that they are bacterial in origin. But these acids are present in relatively high concentrations in the perienteric fluid of freshly collected worms, suggesting that they escape into the keeping medium by diffusion or, perhaps, by excretion. However, these lower acids together account for less than 20% of the total volatile acids present in the perienteric fluid. The residual methylethylacetic acid (46%) is optically active, and the C_6 acid (35%) also appears to be optically active. Optically active isomers of valeric and caproic acids are not known as products of microbial digestion, at any rate of cellulose, nor, so far as we are aware, have they heretofore been discovered as constituents of any biological material. This adds some weight to the opinion that these, and probably the shorter chain acids also, are peculiar products of the metabolism of *Ascaris* itself and not of any bacteria with which it normally is, or experimentally may become, associated.

The results of our analyses, carried out on freshly collected perienteric fluid from worms collected only a few hours before, differ substantially from those recently published by Bueding & Yale (1951), who worked up the acids from saline media in which the worms had been kept:

Percentage of total steam-volatile acids					
C_6	C_5	C_4	C_3	C_2	
36	46	2	4	11	Present work
20	40	2–5	10–15	15–25	Bueding & Yale (1951)

But these differences are less important than those between our respective conclusions regarding the nature of the C_5 acid; for whereas we believe it to consist almost in its entirety of methylethylacetic acid, Bueding & Yale (1951) state that about one-half consists of *n*-valeric acid and the other half of a mixture of *iso*valeric and methylethylacetic acids. Small amounts of an $\alpha\beta$-unsaturated C_5 acid were also reported. We ourselves found traces of a substance which decolorizes bromine water and which might be formic or some unsaturated acid. It is difficult to reconcile our respective conclusions or to explain the differences that exist; but it is conceivable, since much of the C_6 acid has apparently been transferred to the lower fractions in Bueding & Yale's experiments, that these workers were, in fact, dealing with partial degradation products formed—most probably by some kind of microbial activity—from acids which had diffused out of the worms into the culture medium. They themselves deny that bacterial activity was involved in their experiments, on the grounds that the worms used were treated with antibiotic substances. But antibiotics in general are more or less specific in their action, so that these allegedly sterile experiments may reasonably be regarded with some suspicion.

The origin and mode of formation of these acids is wholly obscure. In the past they have often been compared with the lactic acid which can arise anaerobically from carbohydrate, e.g. in muscle and in a variety of bacteria, for it is generally supposed that *Ascaris* itself lives under conditions that approximate fairly nearly to anaerobiosis (see Hobson, 1948). However, the structure of methylethylacetic acid, which contains an asymmetrically branched carbon chain and accounts for practically one-half of the total acids, suggests that the latter can hardly arise from carbohydrate sources. The branched chain of methylethylacetic acid occurs,

however, in isoleucine; it is therefore conceivable that our C_5 acid might arise by oxidative deamination of isoleucine, followed by oxidative decarboxylation of the resulting α-keto acid. These are reactions that are known to occur among vertebrates and probably go on in invertebrates also. Perhaps they are less likely to take place in an animal that leads what appears to be a largely or mainly anaerobic existence, though even here oxidative processes might occur by coupling to simultaneous reductive reactions. But in any case, unless we assume that isoleucine alone is singled out for this kind of treatment we should expect leucine itself to give rise through similar reactions to *iso*valeric acid, and the latter cannot account for more than 10% of the total valeric fraction, according to the spectroscopic evidence. Valine, moreover, would be expected to give rise in a similar way to *iso*butyric acid, whereas our C_4 acid is the *n*-compound. Nor does such a mechanism allow us to explain in any simple manner the relatively large-scale formation of a C_6 acid.

Other mechanisms might be invoked to account for the formation of methylethylacetic acid from isoleucine, the only other commonly occurring biological compound known to contain the appropriate branched chain. We have considered several possibilities of this kind, all of which lead to conclusions that are inconsistent within themselves, like the scheme discussed here. We must at present confess to complete ignorance of the origin, mode of formation and function of these curious compounds.

SUMMARY

1. The steam-volatile fatty acids of the perienteric fluid of *Ascaris lumbricoides* have been analysed by chromatographic methods.

2. Acetic, propionic and *n*-butyric acids have been identified by their chromatographic behaviour and by the piperazinium salt of *n*-butyric acid. Small quantities of formic acid also appear to be present.

3. Infrared spectroscopy showed that the C_5 fraction consists, almost in its entirety, of the optically active methylethylacetic acid (2-methylbutanoic acid). There is a slight preponderance of the laevorotatory form.

4. The C_6 fraction has not so far been identified.

5. It is concluded that these acids are products of the undoubtedly peculiar metabolism of *A. lumbricoides* and are not formed by microbial activity. Their origin, function and mode of formation are unknown.

We wish to thank Dr N. Sheppard for determining the infrared absorption spectra of the acids examined by this method.

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Cacao Polyphenolic Substances

1. FRACTIONATION OF THE FRESH BEAN

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Extremely little is known about the polyphenolic substances of cacao. Numerous analyses have been made but few isolations. To obtain a rational idea of the changes taking place during the fermentation process in the preparation of the bean for the market, the chemistry of the polyphenolic substances present must be placed on a sounder basis. This is only possible by the isolation of the different substances from the fresh bean.

A catechin was isolated by Adam, Hardy & Nierenstein (1931) and by Freudenberg, Cox & Braun (1932), who showed it to be (−)-epicatechin. It is claimed (Adam, 1928) that the epicatechin is present at least in part as a catechin-caffeine complex. Various crude 'tannin' preparations have been isolated (Adam, 1928; Mosimann, 1944). Other substances have been shown to be present by qualitative reactions. The purple colouring matter is due to anthocyanins (Laurence, Price, Robinson & Robinson, 1938) having the colour and solubility reactions of a cyanidin-3-glycoside. Leucoanthocyanins are also present (Knapp & Hearne, 1939).

Numerous proximate methods of determining the 'tannin' of cacao have been developed, few of which have led to any understanding of the substances present. The sum of these studies has shown, however, that the material is a complex mixture of fractions of different solubility. Extractions have been made with ethyl acetate, water (Adam, 1928), neutral buffer, sodium hydroxide, acetic acid (Hallas, 1939), 40% acetone (Duthie, 1938), and dilute hydrochloric acid (Hallas, 1949). The greatest amount determined is about 15% of the dried bean (Hallas, 1939). Of this about two-thirds can be extracted with aqueous acetone, alcohols, or dilute acids, and roughly comprises the fraction studied here. Hallas (1949) has shown that the anthocyanins and leucoanthocyanins in the fresh purple bean are nearly completely soluble in ethanol.

If any success is to be gained in the separation of what is probably a complex mixture of polymers of different degrees of condensation new methods are obviously required. Bradfield, Penney & Wright (1947) and Bradfield & Penney (1948) have had considerable success in separating the simpler polyphenolic substances in tea by partition chromatography on silica gel. White (1949) has shown that the complexity of quebracho tannin extract can be gauged by paper chromatography. Bate-Smith (1949) has demonstrated that anthocyanins and flavones and many similar substances are separable by paper chromatography. A preliminary note (Forsyth, 1949) has been published on the paper chromatography of extracts of cacao beans.

METHODS AND RESULTS

Paper chromatography

Twenty fresh beans were removed from the pod, peeled, and immediately dropped into 100 ml. HCl (0·3N). The acid suspension of cotyledons was homogenized in a Waring Blendor for 3 min. After standing for 15 min. at room temperature (25°) the homogenate was filtered and the extract (20 μl.) spotted on the chromatogram.

The most useful solvent for development was found to be amyl alcohol-acetic acid-water in the proportions 4:1:5. *n*-Butanol-acetic acid (Partridge, 1948) gave similar results, but with cacao extract the spots were not so well separated although they moved much faster. Butanol-acetic acid was used to confirm the identity of the separated substances under the rigid conditions specified by Bate-Smith & Westall (1950). The amyl alcohol-acetic acid chromatograms were run on Whatman no. 1 paper for 18 hr. at 25°. The substances were detected by visible colour and by spraying with ammoniacal $AgNO_3$. Aniline hydrogen oxalate (Partridge, 1949) was used to detect sugars.

Table 1. *Paper chromatogram of a* 0·3N-HCl *extract of fresh Forastero cacao beans*

(Solvent, amyl alcohol-acetic acid-water.)

No. of spot	R_F	Probable nature
7	0·40	Epicatechin
6	0·22	Leucoanthocyanidin
5	0·16	Cyanidin-anthocyanin I
4	0·12	—
3	0·08	Cyanidin-anthocyanin II
2	0·04	—
1	0–0·02	Cyanidin diglycoside leucoanthocyanin

A typical chromatogram showing the main polyphenolic components of an HCl (0·3N) extract of fresh Forastero (purple) beans is shown in Table 1. These substances were invariably present in fresh Forastero beans from many different pods and were the major components.

By extracting the spots from large numbers of papers evidence has been produced (Forsyth, 1949), using Robinson

& Robinson's (1931, 1932) tests, that the components are as stated in the table. Isolation is necessary for better characterization.

As well as the two mobile anthocyanins there was also a trace of anthocyanin not extracted with amyl alcohol (spot no. 1) which had the properties of a cyanidin diglycoside. It hydrolysed to cyanidin and had the solubility of a diglycoside (R_F butanol-acetic 0·17).

Comparison of different beans. Twelve individual beans from the same Imperial College Selection 1 (I.C.S. 1) pod were extracted. They gave identical chromatograms. Twelve individual beans from twelve different I.C.S. 1 pods were extracted. They gave identical chromatograms. Beans of Criollo (non-pigmented) varieties give the same spots except that the anthocyanins are absent. The leucoanthocyanin was present in relatively greater quantity.

The following species and varieties used in chocolate manufacture have been examined and the same spots are present in all the purple beans and only the anthocyanin pigments are missing in the white beans: Red Venezuelan, Nicaragua Criollo, Ecuador Nacional, Trinitario, Calabacillo, *Theobroma pentagona*, *Th. leiocarpa* (Cheesman, 1944). The associated species *Th. bicolor* which is not used contained none of these compounds.

When white, purple-bordered, faint-purple, medium, strong and very strong purple beans were extracted the two anthocyanins were either both absent or both present in the same relative proportion.

Isolation

Imperial College Selection 1 pods were used. It was first necessary to determine what pretreatments of the beans to aid extraction were safe. It was found that fresh beans could be washed in an adapted clothes-washing machine to remove the pulp completely in 2 hr. and then could be heat-killed in boiling water for 5 min. and dried in the oven at 70° in 12 hr. without change in the acid-soluble compounds. In such heat-killed beans the enzymes were completely destroyed. The dried beans were readily peeled and the butter was partly removed by blending with three successive lots of light petroleum (90–100°, 1 ml./g.) at 60°. This procedure removed over 90% of the butter giving a readily extracted stable residue which on extraction gave a chromatogram qualitatively identical to that obtained from fresh beans.

The anthocyanins

Preparation of a concentrate. Attempts to concentrate the anthocyanins via the picrates were unsuccessful, even at concentrations where picric acid crystallized out the pigments remained in the mother liquors. The anthocyanins could, however, be readily concentrated through their lead salts. The method was basically that of Scott-Moncrieff (1930).

Wax-free cacao (100 g.) was blended with 500 ml. 0·3 N-methanolic HCl and left overnight. The extract was filtered off and the process repeated. To the combined extract was added a saturated aqueous solution of lead acetate. The lead chloride which first precipitated was removed and then excess lead acetate added to bring down the pigments as a dark-blue precipitate. After standing overnight the precipitate was filtered off, washed with methanol, and dissolved in the minimum amount of glacial acetic acid (about 100 ml.). This extract was then treated with 2 vol. of ether. The blue lead salt precipitate was washed with ether, dried and ground. The powder was extracted with 25 ml. methanol containing 5% (v/v) HCl and the crude glycosides thrown down as a bright-red gummy mass by addition of 12 vol. ether. The pigments were further purified by extraction with ethyl acetate.

The preliminary purification could also be done simply by ethyl acetate extraction. Wax-free cacao (100 g.) was extracted with 2 × 500 ml. 0·3 N-methanolic HCl as before. The extract was diluted with 2 vol. ether and 4 vol. light petroleum and the crude anthocyanin precipitate was washed successively with light petroleum and ether. It was then extracted exhaustively with ethyl acetate to remove impurities soluble in ethyl acetate.

The simplest method of extraction is with methanol containing 10% acetic acid. After filtration the methanol is removed *in vacuo* and the anthocyanins precipitated by addition of a large excess of ether to the acetic acid. The solid is then washed with ethyl acetate.

Chromatography of the anthocyanin concentrate on columns

Chromatography on alumina, silica gel or kieselguhr did not give good results. The most satisfactory chromatograms were obtained on columns of cellulose pulp (Solka flok 200 mesh, Johnsen, Jorgensen and Wettre Ltd., London). The material, purified as described by Campbell, Work & Mellanby (1951), was packed dry in columns (25 × 3·7 cm.) and washed well first with the aqueous and then with the solvent layer of amyl alcohol-acetic acid.

The anthocyanin concentrate prepared by any of the above methods was taken up in the organic solvent layer of amyl alcohol-acetic acid and added to the column and developed with the same solvent (500 ml.). The column was sucked dry, extruded, and the two individual anthocyanin bands cut out separately and, after being allowed to dry at room temperature, were eluted with methanol containing 0·5% (w/v) HCl. Paper chromatograms of the extracts showed the anthocyanins uncontaminated with any substances reacting with Na_2CO_3, $FeCl_3$, or ammoniacal $AgNO_3$.

The anthocyanins could be precipitated as bright-red gums by the addition of a very large excess of ether to the methanolic HCl extracts from the column. Attempts at crystallization of the small amount of either gum from methanol, ethanol or water containing various amounts of HCl have been unsuccessful. The anthocyanins can be precipitated from methanol solution with ether as dark-purple amorphous powders. These two powders are chromatographically homogeneous and both give cyanidin on hydrolysis with 20% (w/v) HCl, confirmed by paper chromatography in *n*-butanol-2 N-HCl (Bate-Smith, 1949) $R_F = 0{\cdot}70$ and by colour reactions (Robinson & Robinson, 1931). Anthocyanin I is present in about three times the concentration of anthocyanin II as judged by the intensity of the spots.

Anthocyanin I. The R_F value under the standard conditions of Bate-Smith (1949) in butanol-acetic acid is 0·41.

The amorphous powder (10 mg.) was hydrolysed for 30 min. in 0·5 N-H_2SO_4 (0·5 ml.) at 100° in a sealed capsule. The hydrolysate was neutralized with $BaCO_3$, centrifuged and concentrated *in vacuo*. The residue was extracted with methanol and the methanol concentrated to 0·2 ml. This solution gave positive reactions with benzidine and orcinol tests. Paper chromatography showed that two sugars were

present. Spraying with selective reagents (Forsyth, 1948; Partridge, 1948) showed the two spots to be an aldohexose and an aldopentose. The two sugars could not be separated from glucose and arabinose when chromatographed in butanol-acetic acid, phenol (Partridge, 1948), or ethyl acetate-pyridine (Jermyn & Isherwood, 1949).

The pentose was readily separated from added xylose, ribose and lyxose, and the aldohexose readily separated from added galactose and mannose. Quantitative determination of the proportions of the two sugars in the chromatograms (Flood, Hirst & Jones, 1947) showed them to be present in the ratio of 1:1.

That the anthocyanin should be a simple 'cyanidin-glucose-arabinose' with an R_F of 0·41 is very unlikely when compared with the known cyanidin anthocyanins (Table 3). It is most probably acylated. This was confirmed by a brief alkaline hydrolysis for 30 sec. (Robinson & Robinson, 1931). The acyl group was removed and the anthocyanin now had an R_F of 0·35 which is in closer agreement with expectations on theoretical grounds.

Anthocyanin II. The R_F value under standard conditions in butanol-acetic acid is 0·33. The amorphous powder (10 mg.) was hydrolysed with 2N-H_2SO_4 and treated as was anthocyanin I. Chromatography of the sugar solution showed only a single aldohexose to be present which was not separable from glucose in the three solvent mixtures, but was readily separated from galactose or mannose. The anthocyanin was unaffected by brief alkaline hydrolysis, i.e. it is a simple cyanidin monoglucoside.

Non-mobile fraction

On paper chromatography of an HCl or methanolic HCl extract with amyl alcohol-acetic acid much of the material remains on the starting line. This spot contains a trace of an anthocyanin, but also considerable material, which gives cyanidin on boiling with conc. HCl, and reacts with ammoniacal $AgNO_3$.

An anthocyanin concentrate was prepared from wax-free cacao (100 g.) by methanolic-HCl extraction and ether-light petroleum precipitation as previously described. On extracting the anthocyanins from the concentrate with amyl alcohol-acetic acid a considerable residue was left. This residue was dissolved in 0·3N-methanolic HCl (250 ml.) and reprecipitated with ether (2 vol.) three times. The product was then washed with ether and dried to give a brick-red residue which on paper chromatography remained on the starting line.

On boiling with 20% (w/v) HCl a coloration due to liberated cyanidin (R_F in butanol-2N-HCl, 0·69) was produced. Hydrolysis with 2N-H_2SO_4 followed by neutralization with $BaCO_3$ gave a sugar solution, shown by chromatography to contain glucose and arabinose in the proportion of roughly 3:1.

The ethyl acetate fraction

Wax-free cacao (100 g.) was extracted with two successive litre lots of 0·3N-HCl. The filtered extract was shaken with three successive 500 ml. lots of ethyl acetate. The ethyl acetate was dried (Na_2SO_4), the solvent removed under reduced pressure (nitrogen bubbler) and the residue transferred in boiling $CHCl_3$ to a sintered-glass funnel, washed with $CHCl_3$ and dried. About 3 g. of a light-tan coloured amorphous powder were obtained containing the substances which give '$AgNO_3$-positive' spots on the paper chromatograms. The extraction with $CHCl_3$ had no effect on the pattern of any of the spots, showing that by this method of extraction no catechin-caffeine complex was present.

The powder was taken up in a small volume of the top layer of the amyl alcohol-acetic acid-water mixture and transferred to a cellulose pulp column (12·5 × 3·7 cm.) and the column developed with the same solvent. The eluate was collected in 2 ml. lots and spotted on paper chromatograms. Suitable cuts were made and similar fractions combined. Each fraction was then taken to dryness *in vacuo* and the residue washed with $CHCl_3$. The results are shown in Table 2.

Cut A. Attempts to obtain any crystalline products from cut *A* were unsuccessful. On paper chromatography a series of fast-moving brown and yellow streaks were observed. No spot was detected on spraying with aqueous NaCN or vanillin-HCl (Bradfield *et al.* 1947), or with acetic acid-

Table 2. *Fractionation of the ethyl-acetate solubles* (3 *g.*) *on a cellulose column with amyl alcohol-acetic acid*

Cut	Vol. eluate (ml.)	Dry wt. (mg.)	Components provisionally identified	R_F Amyl alcohol-acetic acid	R_F Butanol-acetic acid	R_F Phenol-water
O	40	—	—	—	—	—
A	34	90	*o*-Hydroxyphenols absent	0·75–0·95	—	—
B	48	1028	(−)-Epicatechin	0·40	0·65	0·48
			Catechin	0·50	0·74	0·36
C	26	304	(−)-Epicatechin	—	—	—
			Gallocatechin	0·27	0·57	0·25
D	78	578	(−)-Epicatechin	—	—	—
			Gallocatechin	—	—	—
			Epigallocatechin	0·22	0·48	0·32
			Leucoanthocyanidin	0·19	0·40	—
E	98	169	Leucoanthocyanidin	—	—	—
			Epigallocatechin	—	—	—
			Spot no. 4 (Table 1)	0·12	0·44	0·21
F	144	84	Spot no. 4 (Table 1)	—	—	—
			Trace of anthocyanin I	—	—	—
G	200	22	Spot no. 2 (Table 1)	0·04	0·39	0·12

ammonium molybdate (Quastel, 1931), i.e. no catechins or other *o*-hydroxyphenols were present.

Cut B. Cut *B* consisted mainly of the major catechin (spot no. 7 in Table 1) with traces of another compound. Both substances were catechins since they could be detected by spraying with ammoniacal $AgNO_3$, $FeCl_3$, Na_2CO_3, ammonium molybdate, NaCN and vanillin-HCl solutions.

The powder from cut *B* crystallized from water. The major catechin was the more insoluble and the material was recrystallized till the crystals were chromatographically homogeneous. In all 722 mg. of colourless short prisms were obtained, m.p. 237° (uncorr.); $[\alpha]_D^{20°} - 58°$ ($c = 2$ in aqueous acetone, 1/1). (Found: C, 49·6; H, 6·2. Calc. for $C_{15}H_{14}O_6 . 4H_2O$: C, 49·7; H, 6·1.) The acetyl derivative was prepared, m.p. 151°.

The pure crystalline material was responsible for spot no. 7 on the chromatograms. It had the R_F values shown in Table 2. It gives an intense green colour with ferric salts and a heavy reddish brown precipitate with boiling HCl. All the above properties show the substance to be (−)-epicatechin. This has previously been isolated from cacao by ether extraction (Freudenberg *et al.* 1932). A preparation isolated in the conventional manner showed identical properties with the above.

The mother liquors from the crystallization of (−)-epicatechin were combined. A large excess of (−)-epicatechin was still present. The catechins could be purified and partly separated by simply adding the aqueous solution to a column of wet cellulose pulp (12·5 × 3·7 cm.) and eluting with water. After 30 ml. of blank eluate had passed through 34 ml. was collected containing only coloured impurities followed by 120 ml. of a mixture of catechins and then 130 ml. containing only (−)-epicatechin. The catechin fractions were exhaustively extracted with ether and the ether evaporated. (−)-Epicatechin (67 mg.) and 72 mg. of the mixture were obtained. The mixture gave on crystallization from water mainly short prisms with a few long needles. Paper chromatography suggested that (−)-epicatechin and the other catechin were present in the mixture in the ratio 4/1 as judged from the intensity of the spots. Thus the maximum possible yield of the minor catechin would be 14·5 mg. Isolation in the presence of excess (−)-epicatechin was not successful. Incubation of the material with a dried preparation of mycelium of *Aspergillus niger* for 12 days failed to liberate any gallic acid and the chromatogram was unchanged. The same preparation readily split an authentic sample of (−)-epigallocatechin gallate (Bradfield *et al.* 1947). The substance is not, therefore, a catechin gallate ester. It was also stable to mild acid hydrolysis. From R_F values (Bradfield & Bate-Smith, 1950; Wender & Gage, 1949) the substance is provisionally identified as catechin itself. The rotation is not known.

Cut C. Paper chromatography showed that cut *C* consisted almost entirely of (−)-epicatechin. On crystallization from water 234 mg. of (−)-epicatechin were obtained. The mother liquor on concentration showed traces of another catechin which gave all the catechin reactions and was not a gallate ester. It could not be separated by paper chromatography from an authentic sample of (±)-gallocatechin. The substance was obviously present in too small quantity for isolation to be possible.

Cut D. From cut *D* 578 mg. of a light-brown powder was collected. On refluxing with 20% (w/v) HCl, or better ethanolic HCl, cyanidin is produced, confirmed by paper chromatography in butanol-2N-HCl and by colour tests.

The powder was very soluble in water. On paper chromatography of the solution, it was seen to be a mixture of (−)-epicatechin, gallocatechin, and spot no. 6 of Table 1. The powder was dissolved in 5 ml. methanol and precipitated with 45 ml. ether. The ether-soluble fraction was evaporated to give 145 mg. of a colourless solid. This material did not give cyanidin on treatment with HCl. Paper chromatography showed (−)-epicatechin, gallocatechin, and a third spot giving all the catechin reactions to be present. The mixture has not been separated. The chromatogram was unaffected by tannase. The unknown catechin could not be separated from (−)-epigallocatechin on chromatography. The three catechins were judged to be present in the proportion 3:1:3 from the intensity of the spots.

The ether-insoluble fraction was a light-brown amorphous powder (400 mg.) which could not be obtained crystalline and which darkened on heating, but did not melt below 300°. This material gave cyanidin on treatment with strong acid, but no sugar could be detected on milder hydrolysis. It did not give an immediate spot with ammoniacal $AgNO_3$ but only on standing.

Cut E. Cut *E* was treated as was cut *D*, 65 mg. were ether-soluble. The ether-insoluble material was the leucocyanidin as before (100 mg.). The ether-soluble material was a mixture of epigallocatechin and spot no. 4 of Table 1.

Cut F. Cut *F* consisted mainly of spot no. 4 in Table 1. It gave the usual catechin colour reactions and was unaffected by tannase. No crystalline products could be obtained.

Cut G. Cut *G* contained very little material which had the properties of spot no. 2 in Table 1. It gave the catechin colour reactions, but was unaffected by tannase.

DISCUSSION

The fresh cacao bean is seen to contain a very complicated mixture of polyphenolic substances. At least eleven different compounds can be distinguished by chromatography. The similarity between widely different varieties is striking.

The two main anthocyanins have been shown to contain respectively, glucose and arabinose, and glucose, we would therefore expect them to be cyanidin-3-arabinoglucoside and cyanidin-3-glucoside, i.e. chrysanthemin. However, the second anthocyanin is certainly not chrysanthemin (solubility of picrate), and an examination of colour reactions would suggest that the sugars are linked to a different hydroxyl (Robertson & Robinson, 1929).

To settle this point it would be necessary to isolate the pigments in large yield and compare them with synthetic preparations. Although up to 50% of the naturally occurring cyanidin glycosides may be of the 'pentose' glycoside (including methyl pentose) form (Robinson & Robinson, 1931, 1932) it is seldom known which pentose is present, and it is usually assumed that the pentose is rhamnose. An exception is sambucyanin (Nolan & Casey, 1931), which contains an aldopentose and glucose. Arabinose does not appear to have been identified previously in a cyanidin glycoside, although

Table 3. *Comparison of cacao, and known cyanidin anthocyanins*

Anthocyanin	Sugars	R_F Butanol-acetic acid	Colour at pH 8	Sugar linkage
Mekocyanin	Gentiobiose	0·29	Brown-red	3
Cacao anthocyanin II	Glucose	0·33	Blue-violet	?
Chrysanthemin	Glucose	0·33	Brown-red	3
Mildly hydrolysed anthocyanin I	Arabinose-glucose	0·35	Blue-violet	?
Antirrhinin	Rhamnose-glucose	0·37	Brown-cherry	3
Cacao anthocyanin I	Arabinose-glucose	0·41	Blue-violet	?
Cyanidin	—	0·69 (in butanol/2N-HCl)	Blue-violet	—

vicianose is a common constituent of other types of glycosides. The available information is summarized in Table 3.

At least two compounds of the leucoanthocyanin class are present, both based on cyanidin. One would appear to be a leucocyanidin and the other (no. 1) a leucocyanidin glycoside. The second is probably a mixture of different glycosides based on both arabinose and glucose. Attempts are being made to isolate the leucocyanidin in large yield for structural investigation.

At least six of the polyphenols have the properties of catechins of which the only one present in quantity (at least 40% of the total ethyl acetate-soluble 'tannins') is the previously isolated (−)-epicatechin. No evidence of gallates was encountered. A comparison of the catechins isolated from cacao and tea (Bradfield *et al.* 1947; Bradfield & Penney, 1948) has led to the provisional identification of catechin, gallocatechin, and epigallocatechin, by chromatography. The other two catechins are unidentified.

SUMMARY

1. The polyphenolic components in extracts of fresh cacao beans have been examined by paper strip chromatography.

2. At least eleven polyphenols are present and have been separated on columns of cellulose pulp.

3. In Forastero (purple) beans there are two main anthocyanins with also a trace of a third pigment, with the properties of a cyanidin diglycoside. The two main pigments are a cyanidin monoglycoside and a cyanidin pentose glycoside, the sugar radicals being respectively glucose and glucose-arabinose.

4. A sugar-free leucocyanidin and a mixture of leucocyanidin glycosides are also present.

5. There are at least six catechin-like substances of which epicatechin is the main component. From chromatographic comparison, catechin, epigallocatechin and gallocatechin, would appear to be present.

6. The chemical similarity between different species and varieties of beans used in chocolate manufacture is striking.

I am indebted to Dr A. E. Bradfield for supplying the samples of gallocatechin, epigallocatechin, and epigallocatechin gallate which were used in the chromatographic work, to Dr A. C. Thaysen, Director, for his interest in this work, and to the Colonial Products Research Council for permission to publish.

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Cacao Polyphenolic Substances

2. CHANGES DURING FERMENTATION

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Fresh cacao beans are prepared for marketing by heaping in boxes so that their covering of mucilaginous pulp can be broken down through the action of yeasts and acetic acid bacteria (Knapp, 1937). After this fermentation the beans are sun-dried. During both processes various changes take place in the cotyledons of the beans, which result in a product suitable for the manufacture of chocolate. The most striking chemical changes taking place during processing of fresh cacao beans are the alterations in the polyphenolic substances of the beans, the simpler polyphenols yielding insoluble complexes (Hallas, 1939, 1949).

From analogy with tea fermentation, it has generally been assumed (Knapp, 1937) that the initial change in the cacao bean fermentation is one of oxidation by atmospheric oxygen activated by a polyphenol oxidase system. Oxidases are certainly present in abundance (Brill, 1915; Ciferri, 1931).

In previous work in this series (Forsyth, 1949, 1952) it has been shown that the polyphenols extracted from fresh Forastero cacao with dilute acid consist mainly of catechin and cyanidin compounds. Both anthocyanins and leucoanthocyanins are present. Of the catechins, epicatechin is present in by far the greater proportion.

By quantitative paper chromatography of extracts of beans removed from the fermentation heap throughout the process, the destruction and conversion of the main polyphenols has now been followed. The changes during artificial treatments of the beans have also been investigated.

METHODS

Commercially fermented beans. Twenty beans were taken at random from a fermentation 'sweat box'. This number is required to give a representative sample. The beans were peeled and the cotyledons (about 20 g.) immediately extracted with 0·3 N-HCl as described in Part 1 (Forsyth, 1952).

Chromatographic estimation of polyphenols. The extract (0·2 ml.) was streaked across the starting line (16 cm.) of a Whatman no. 1 paper sheet 20 cm. wide. Two lanes at the side were used to spot on marker solutions of fresh bean extract. The sheets were then chromatographed with amyl alcohol-acetic acid-water for 18 hr. (Forsyth, 1949, 1952) by descending chromatography. Before drying the paper the anthocyanin pigments were cut out and extracted to pre-

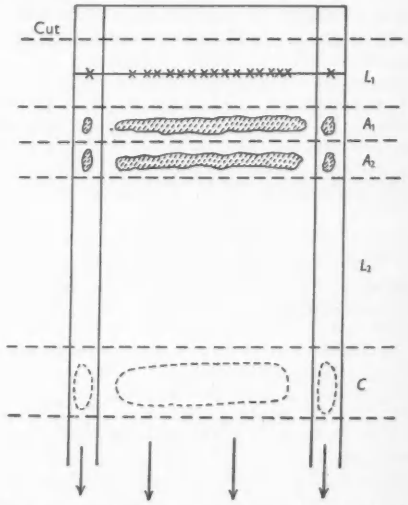

Fig. 1. Quantitative paper chromatography of 0·3 N-HCl extract of fresh cacao beans. L_1, complex leucocyanidins; L_2, leucocyanidin; A_1, cyanidin monoglucoside; A_2, cyanidin arabinoglucoside; C, epicatechin.

vent fading. The remainder of the paper was allowed to dry at room temperature. The marker strips were cut out and passed through vanillin-HCl to detect the epicatechin spot. The sheets were cut as shown in Fig. 1. In all, five components were estimated which make up the bulk of the soluble polyphenols of Forastero cacao beans.

The leucocyanidins (L_1, L_2). These compounds were not detected on the paper, but were known to be present in the selected sections of the chromatogram (Forsyth, 1952), along with traces of the other catechins. They were estimated by refluxing the paper sections for 15 min. with 10 ml. *n*-butanol and 2 ml. 10 N-HCl which converts them to cyanidin. The cyanidin solution was purified and determined colorimetrically as described by Hallas (1949). Blank paper sections gave no coloration with this treatment.

The anthocyanins (A_1, A_2). These pigments were extracted from the paper with 10 ml. methanolic HCl (0·3 N) and estimated colorimetrically (Hallas, 1949).

(−)-*Epicatechin* (*C*). The catechin was detected with vanillin-HCl and estimated by extraction with 10 ml. 1% (v/v) H_2SO_4 and titration with 0·01 N-$KMnO_4$.

As controls, fresh beans were used, extracted and chromatographed simultaneously and in an identical manner. All results are expressed as a percentage of each component as present in the fresh bean. An HCl (0·3 N) extract is stable for at least 24 hr. since, although the enzymes do not appear to be destroyed by the acid, they do not act at this low pH.

Laboratory treatment of beans for the study of oxidase activity

Grinding in air. When fresh, peeled beans are ground in a mortar and exposed to the air a rapid browning and complete disappearance of all the soluble polyphenols present takes place within 1 hr. After only 15 min. grinding over 80% of each component has been destroyed. Such a treatment is not suitable for enzyme studies. It was found, however, that similar extensive changes take place when beans are disintegrated in water, if sufficient aeration is permitted.

Grinding in aqueous solutions. Twenty fresh peeled beans were disintegrated in a Waring Blendor for 1 min. with 100 ml. water, left for 4 min., reblended for 0·5 min., left for 4·5 min., reblended for 0·5 min., left for 4·5 min., and then the acidity of the suspension made 0·3 N with conc. HCl and blended for a further minute. The final acid suspension was filtered and the polyphenols remaining determined in this filtrate by the previously described method. In this way, the beans were exposed for 15 min. to high aeration and disintegration. Boiled beans treated similarly showed no change. The pH during the blending could be made any desired value, and enzymic poisons could be introduced, to study their effect on the oxidase system. To study the effect of temperature the beans could also be blended in hot water, maintained at 50° by immersing the blender in a water bath between the short blends.

Determination of oxidase activity in whole beans. Although cacao beans contain a particularly powerful oxidase system the results from a commercial fermentation did not suggest that this system is acting in the cotyledons. This could only be due to the absence of an adequate supply of oxygen gaining access to the cotyledons during commercial fermentation. To test this, fresh pulp-free beans were fermented in anaerobic jars packed with cellulose pulp containing buffer pH 5·5 for 5 days at 48°, conditions similar to those in a sweat box but under completely sterile conditions, and in a hydrogen atmosphere.

The beans were extracted with acid in the normal manner, but the polyphenols in the extract from both peeled and unpeeled beans were estimated to determine the losses due to exudation of polyphenols, from the cotyledons to the tests.

Laboratory treatment of beans for the study of anaerobic enzyme activity

The above methods are not very suitable for a more intensive study of the enzymes since it would be difficult to obtain a disintegrated suspension of the beans without oxidation taking place. It was therefore decided to investigate whether a dry powder could be obtained containing the enzymes and substrate intact. This is feasible, since in fresh beans all the polyphenolic substances are located in the vacuoles of special isolated cells, and if the beans are washed free of pulp and dried in the sun, or dehydrated at low temperatures in a well ventilated oven, the cells are dehydrated *in situ*, and no change in the solubility of the components can be detected. The dry beans can then be ground to a fine powder without enzyme action taking place owing to their low moisture content. It was found that such a powder serves as a reasonably stable source of enzyme and substrate.

To investigate the anaerobic changes dry powder (20 g.) was submerged in water (100 ml.) sealed with liquid paraffin, for various times. The powder wetted easily and only a preliminary shake to mix was given. The suspension was then acidified to 0·3 N with HCl as before, blended for 3 min., and filtered. The polyphenols were estimated in the usual manner. The pH and temperature could be varied and enzyme poisons introduced. In no case was the catechin affected under anaerobic conditions, except where H_2O_2 was added, and the constancy of the catechin value was used as a check on the anaerobiosis. Any extract in which the catechin deviated from the control by more than 5% was discarded. Boiled beans were unaffected by this treatment.

RESULTS

In normal Trinidad practice the fresh beans are allowed to ferment in sweat boxes for from 6 to 8 days and are then removed to the drying platform for sun-drying. A typical analysis of such beans is shown in Fig. 2.

The effects of pH and of inhibitors on the changes activated by the oxidases, during blending, are shown in Table 1. When the beans were ground at 50° at pH 5·0 the oxidase system remained active, i.e. the oxidases were not to any great extent inhibited by the highest temperature and acidity reached in commercially fermented beans in the sweat box. However, when the residual epicatechin was determined in both the cotyledons and testa of whole unpeeled beans, there was no significant difference in amount between the catechin remaining in the cotyledons of beans, fermented for 5 days in the complete absence of external oxygen, and those commercially fermented. The loss of catechin (20–30%) must be due in both cases to exudation

from the cotyledons to the testa. This exuded catechin becomes partly oxidized in the testa of commercial beans but not, of course, in beans fermented under completely anaerobic conditions.

Anaerobic enzyme activity

That the dry powder used in the following experiments can serve as a source of enzymes and substrate comparable with the fresh beans is shown in Table 2. In this table is also included a test for peroxidase which is shown to be present but which does not act under anaerobic conditions unless hydrogen peroxide is added.

It will be seen in Table 2 that some changes take place in the cyanidin compounds under anaerobic conditions even at room temperature. These changes are much more marked at 48°. This temperature was chosen as being within the range of temperature prevailing in the sweat box at the time of killing of the bean. The result of incubating the powdered beans anaerobically at 48° at various pH values are shown in Figs. 3 and 4.

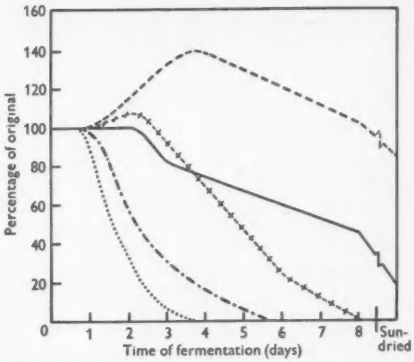

Fig. 2. Changes in soluble polyphenols during a commercial fermentation. Symbols as in Fig. 1; ....., A_1; -·-·-, A_2; ——, C; - - - -, L_1; -+-+, L_2. Sun-dried refers to beans which, after undergoing the commercial fermentation, were exposed to the sun till dry, i.e. the commercial product as shipped.

Table 1. *Oxidase activity of fresh bean homogenates in water, various buffers (MacIlvaine's) and in the presence of inhibitors*

(For conditions of blending and method of estimation see text. The inhibitor was added before disintegration of the beans. Results as percentage of original amount of each component remaining after 15 min.)

Solution	pH	Cyanidin monoglucoside	Cyanidin arabino-glucoside	Complex leuco-cyanidins	Leuco-cyanidin	Epicatechin
Water	—	<5	<5	17	<5	13
	5·5	<5	13	7	<5	18
MacIlvaine's buffer	5·0	10	8	12	<5	21
	4·5	12	10	16	10	35
	4·0	55	54	58	72	80
10^{-2} M-KCN	5·5	52	50	80	60	71
10^{-2} M-KCN	5·5	103	107	100	94	101
10^{-1} M-Ascorbic acid	5·5	72	67	101	74	102

Table 2. *Comparison between fresh beans, sun-dried beans, and sun-dried powdered beans*

(For methods of estimation see text. Results as percentage of each component in original fresh beans.)

Test for	Preliminary treatment	Cyanidin mono-glucoside	Cyanidin arabino-glucoside	Complex leuco-cyanidin	Leuco-cyanidin	Epicatechin
Substrate	Sun-dried beans	91	94	110	97	94
	Sun-dried powdered beans	108	110	92	105	101
Oxidase activity	Fresh beans, blended water for 15 min. at 25°	<5	<5	15	<5	11
	Dry powdered beans blended as with fresh	<5	<5	32	<5	21
Anaerobic enzymic activity	Fresh beans killed by freezing and incubated in water anaerobically at 25° for 18 hr.	55	78	107	97	99
	Dry powdered beans treated as with frozen beans	45	87	115	87	102
Peroxidase	Dry powdered beans incubated anaerobically as above in 0·1 M-H_2O_2	33	69	59	42	58

Whole beans killed by preliminary freezing showed the same changes when incubated anaerobically as do powdered beans. The pH of 6·2 was chosen as being about the pH prevailing at the time of the death of the bean and pH 5·5 as covering the latter part of the fermentation.

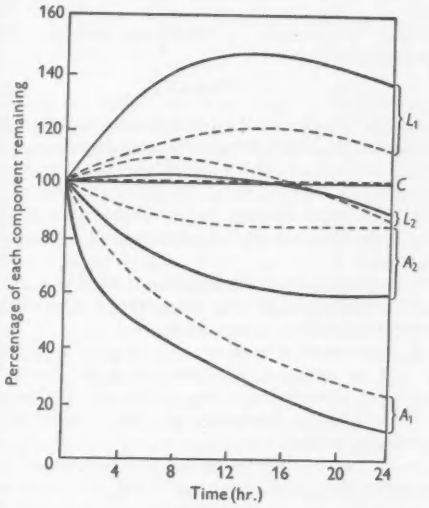

Fig. 3. Conversion of polyphenols in sun-dried powder at 48° under anaerobic conditions. Symbols as in Fig. 1; ——, pH 6·2; – – –, pH 5·5. 20 g. powder suspended in 100 ml. MacIlvaine's buffer under a paraffin seal for various times and the changes estimated as described in the text.

The effect of inhibitors on the anaerobic reactions is shown in Table 3. Sodium fluoride and copper sulphate appear to inhibit the formation of the complex leucocyanidins (L_1) while not preventing the apparent conversion of the anthocyanins to leucocyanidin (L_2).

In no case was cyanidin itself found on any of the chromatograms indicating that a simple hydrolytic mechanism was unlikely.

DISCUSSION

It has been generally assumed that the main change in the cacao polyphenols during fermentation is one of oxidation by a polyphenol oxidase system requiring external oxygen.

The beans certainly contain a particularly powerful polyphenol oxidase system capable of removing over 80 % of the total polyphenols in 15 min. when fresh beans are disintegrated and strongly aerated in buffer solutions, at the temperature and acidity prevailing in the sweat box. Peroxidase is also present. The oxidase has a high resistance to potassium cyanide in common with tea polyphenol oxidases (Lamb & Sreerangachar, 1940). However, during the commercial fermentation of the whole cacao beans no such rapid removal of the polyphenols takes place. Instead the simple cyanidin compounds are destroyed and more complex leucocyanidins are formed. The catechin is slowly removed from the cotyledons. When powdered beans are fermented under anaerobic conditions similar changes take place in the cyanidin compounds, but the catechin is completely unaffected. By fermenting whole beans under anaerobic conditions, it can be shown that the loss of catechin in commercial fermentation is due entirely to its exudation from the cotyledons to the testa.

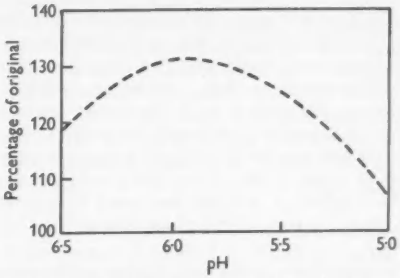

Fig. 4. The percentage of the complex leucocyanidin fraction (L_1) remaining after incubation of sun-dried powder at various pH values in MacIlvaine's buffers anaerobically for 5 hr. at 48°. (Amounts used as in Fig. 3.)

Table 3. *Effect of inhibitors on the anaerobic enzymic activity of sun-dried powdered beans*

(Powder (20 g.) was suspended in MacIlvaine's buffer pH 6·0 (100 ml.), sealed with liquid paraffin and maintained at 48° for 18 hr. then estimated as described in text.)

	Cyanidin mono-glucoside	Cyanidin arabino-glucoside	Complex leuco-cyanidins	Leuco-cyanidin
Boiled solution	92	95	100	98
Buffer	20	70	150	104
NaF (10^{-2} M)	15	64	72	130
$CuSO_4$ (10^{-2} M)	33	62	58	132
KCN (10^{-2} M)	30	67	115	96

The lack of activity of the oxidase system in the cotyledons during a commercial fermentation can only be due to a restriction in the supply of available oxygen. Such lack of oxygen is not surprising. In fermenting beans the microflora of the pulp will most certainly consume most of the oxygen introduced into the heap by ventilation. This is supported by the observation that fermenting pulp decolorizes methylene blue. Further, even in pulp-free aseptically fermented beans, the cotyledons resist oxidation. This is due to the changes which take place in the beans about the second day, when they are killed by the temperature and acidity. The free space between the cotyledons and the testa then becomes completely filled with a continuous layer of purplish juice. Paper chromatography shows that this juice contains all the soluble components of the cotyledons and that it is especially rich in epicatechin. In a commercial fermentation this juice is partly absorbed by the testa and some is even lost in the pulp and sweatings. The exudate and the testa themselves undergo browning during fermentation, but even in fully fermented beans the juice still contains considerable catechin and acts as a further barrier of oxidizable substances and prevents access of air to the cotyledons. Oxidation in the cotyledons (i.e. the part used in chocolate manufacture) only takes place during the drying period.

The significant change during fermentation would thus appear to be the conversion of the cyanidin compounds into more complex products by an enzyme system acting independently of external oxygen. This conversion has an optimum pH of about 6·0, the mean pH of the fermenting bean on the first day after the death of the bean. When the reaction is carried out in the presence of enzyme poisons the conversion of pigments to leuco compounds and the subsequent condensation of the latter can be differentiated.

It is tempting to suggest that this conversion of cyanidin compounds may be of greater importance than the previously postulated 'oxidation of tannins' in determining the flavour and aroma of the final product.

SUMMARY

1. The changes in the polyphenolic constituents of cacao cotyledons during commercial fermentation have been estimated by quantitative paper chromatography.

2. The main change is the conversion of the simple cyanidin compounds to more complex leucocyanidins.

3. Although oxidases are present they do not act in the cotyledons during fermentation due to the anaerobic conditions prevailing.

4. The catechin is partly lost by exudation and is then to some extent oxidized under the more aerobic conditions prevailing in the testa. Oxidation in the cotyledons, however, only takes place during the drying period.

5. It is possible to obtain conversion of the cyanidin compounds with sun-dried, unfermented, powdered beans, in buffers under anaerobic conditions. The reaction appears to consist of several stages.

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Distribution of Copper and Zinc in Mammalian Eyes. Occurrence of Metals in Melanin Fractions from Eye Tissues

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Several methods have been used in the investigation of trace elements in eye tissues. Burdon-Cooper (1928) and Burdon-Cooper & Lewis (1929) give the results of emission spectrographic analysis of the mineral constituents of normal and cataractous human-eye lenses. The technique failed to demonstrate the presence of zinc, iron and manganese, which Tauber & Krause (1943) were later able to detect (and estimate) by other methods. The latter authors pointed out that the persistent spectral lines of the three elements could easily be obscured by lines due to elements such as calcium and phosphorus, which are present in the ash as major constituents.

The first stage in the present investigation was the application of modern methods of emission spectrum analysis to the problem of detecting and determining trace metals in eye tissues (A.L.S. and M.H.S., see Shakir, 1948). So far as detection was concerned, excellent results were obtained, but the range covered by zinc, copper and manganese contents of various eye tissues proved to be too narrow for emission spectra to give quantitative information of sufficient accuracy. Accordingly, a good deal of exploratory work was carried out using organic reagents forming metallic complexes which could be estimated by spectrophotometric methods. Selected procedures have now been improved and the results revised and extended (J.M.B.).

In the course of the work based on emission spectroscopy, semi-quantitative estimations of trace elements were made on dialysed and undialysed tissues from fish eyes (Shakir, 1948). The presence of comparatively large quantities of dialysable inorganic material results in a cloud of vaporized salts around the electrodes, and this has a marked effect on the recorded intensities of the spectral lines shown by the 'trace' elements. This effect introduces uncertainty into the validity of comparing the results with dialysed and undialysed preparations. A fivefold difference in trace metal concentration is certainly demonstrable, but it is difficult to be sure about small differences. The emission spectrum technique is thus not sufficiently sensitive for further study of the rather small differences recorded by Tauber & Krause (1943) in respect of the concentrations of zinc, copper and manganese in cattle-eye tissues. In spite of such limitations, it seemed clear that tissues from fish eyes contained non-dialysable zinc and copper in excess of the retained amounts of other trace metals. The preliminary work with dithizone zinc and copper complexes indicated that it might be possible to establish significant differences between the various eye tissues in mammals. Repetition and extension of the work shows this to be the case.

Leiner & Leiner (1944) had shown for zinc in fish eyes that such differences occur. Although the absolute concentrations found in each tissue varied greatly from species to species, a list showing the different types of tissues in order of decreasing zinc content was practically the same for all the fishes studied.

The results of Tauber & Krause (1943) on cattle eyes do not show a similar distribution of zinc, but the analytical method described in their paper seems inadequate. Their determinations of copper concentrations in eye tissues appear to be the only ones recorded in the literature.

It was therefore decided that the whole problem of zinc and copper in eye tissues deserved further study. The first part of this paper embodies the experimental details and results of determinations of zinc and copper concentrations in all the eye tissues of several mammals. The results of these determinations show that the iris and choroid tissues contain the highest concentrations of zinc and copper. It therefore became necessary to investigate the more precise location of these metals in irises and choroids. The second part of the paper deals with the experimental methods and results of a fractionation of irises, and a study of a pigment fraction obtained from irises and choroids.

1. ZINC AND COPPER CONCENTRATIONS IN THE VARIOUS EYE TISSUES

Experimental

Materials

Cattle and sheep eyes were obtained from the abattoir and dissected within 12 hr. of the death of the animals.

Whale eyes were obtained from sperm whales caught in the Antarctic region, and had been stored (whaling ship

'*Balaena*') in tins at a temperature below 0° for several months before dissection.

Rabbit eyes from animals of Dutch, cross-bred, Himalayan and albino varieties, were obtained through the courtesy of Dr A. U. Smith, from the National Institute for Medical Research, Mill Hill, London, N.W. 7, and were dissected 2–3 days after the death of the animals.

Analytical methods

General. Concentrations of Zn and Cu were determined mainly on a dry weight basis, for the tissues can be lightly washed, to remove adhering fluids and particles, with very little alterations in solid content, though the natural wet weight cannot then be accurately determined. Approximate wet weights of sheep and cattle tissues were obtained by drying the tissues, after washing, with ashless filter paper. A comparison of wet and dry weight concentrations of the trace metals then enables the effect of the loss of water from liquid tissues, such as the vitreous humour, to be gauged.

Preparation of tissues for analysis. Dissection was carried out with stainless steel instruments. An incision in the sclera was made with a scalpel and the front portion of the eye parted from the back by cutting round the outer margin of the iris. The tissues of the two portions were then separated and the two parts of the sclera combined, after removing the adhering muscle and fatty tissue. Each individual tissue was then washed with twice-distilled water. All manipulations after washing were carried out with glass rods.

The tissues were dried to constant weight in an oven maintained at 110°.

Tissues were normally incinerated in translucent silica crucibles at 450–550° in an electric muffle furnace with a silica lining. If the last trace of carbon residue was difficult to burn away it was found that addition of a few drops of twice-distilled water to the cooled ash permitted easy oxidation of the carbon on reheating. Lens tissue must be very slowly heated to 450°, otherwise the contents of the crucible froth over.

To each crucible containing ash, 0·1 N-H_2SO_4 (10 ml.) was added and the contents evaporated to dryness on a steam bath. More 0·1 N-H_2SO_4 (5 ml.) was then added and the crucible heated on the steam bath for a further 10–15 min. The contents were then washed into the beaker used to support the crucible in the extraction procedure, and the solution was made up to a standard volume ready for the estimation of Cu.

Analytical method. Both Cu and Zn were estimated colorimetrically by means of diphenylthiocarbazone (dithizone) solution in CCl_4. From an acid solution only the Cu complex is extracted; if this is first removed, Zn can then be estimated by buffering to pH 4·75, and again extracting with dithizone. Extinction coefficients of the CCl_4 solutions were measured by means of a Beckman photoelectric spectrophotometer.

The method given by Sandell (1944) was used for Cu, and a modification of the method of Vallee & Gibson (1948) for Zn.

The absorption curve of dithizone in CCl_4 solution (Fig. 1) has a minimum about 510 mμ., and at this wavelength the curve for copper dithizonate (Fig. 2) is near its maximum. Thus, extraction with a known excess of dithizone, and measurements of the extinction coefficient at 510 mμ., will indicate the amount of Cu present. This method is not practicable for Zn because a large excess of dithizone is needed for the Zn to be extracted quantitatively. The extinction coefficient of pure zinc dithizonate at 620 mμ. is very low indeed (Fig. 3) and thus, in a solution containing both dithizone and zinc dithizonate, measurement of the E value at 620 mμ. gives a measure of the excess dithizone present. Zinc dithizonate has a maximum at 535 mμ. (Fig. 3) and thus, if the extinction at this wavelength is measured, and the contribution made by excess dithizone to the total absorption at 535 mμ. is calculated and subtracted, the Zn can be estimated. (The relative E values for pure dithizone at 620 and 535 mμ. are known (Fig. 1), so that measurement at 620 mμ. allows the E value at 535 mμ. to be computed).

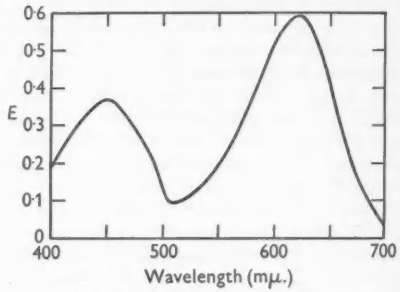

Fig. 1. Absorption spectrum of diphenylthiocarbazone in CCl_4. $E^{1\%}_{1\,cm.}$ at $\lambda_{620\ m\mu.} = 540$.

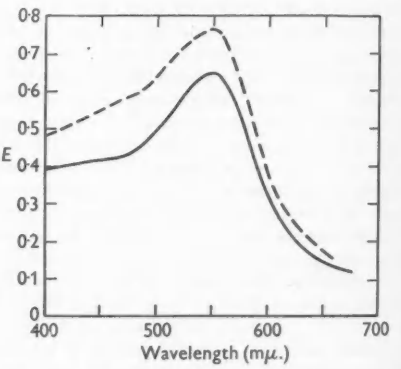

Fig. 2. Absorption spectra of copper dithizonate solutions in CCl_4. — — —, ash from eye tissues dissolved in 0·1 N-H_2SO_4. Excess of this solution extracted with dithizone in CCl_4. ——, excess $CuSO_4$ in 0·1 N-H_2SO_4 extracted with dithizone in CCl_4 (about 0·1 μg. Cu/ml.).

Reagents and apparatus. Twice-distilled water (the final distillation being from, and into, Pyrex glass vessels) was used to make up all solutions, and for the final washing of the vessels. All glass vessels used were of Pyrex and, after washing, were dried in an oven at 110° and stored away from dust.

All reagents used were of standard A.R. grade except diphenylthiocarbazone (British Drug Houses Ltd.) which bore no special label:

(*a*) 0·1 N-H_2SO_4: 2·7 ml. A.R. H_2SO_4 made up to 1 l.

(*b*) Sodium thiosulphate solution: 25 g. $Na_2S_2O_3.5H_2O$ in 100 ml. water.

(*c*) Buffer (pH 4·75): 2 N-acetic acid (500 ml.) and 2 N-sodium acetate (500 ml.) were mixed together. Even when reagents of A.R. quality were used, this solution gave a substantial 'blank' reading in the Zn estimation. This was eliminated by shaking 1 l. of the buffer solution, for 5–10 min. each time, with successive portions of 0·01 % dithizone solution, and discarding the lower (CCl_4) layers until the colour of the dithizone remained unchanged.

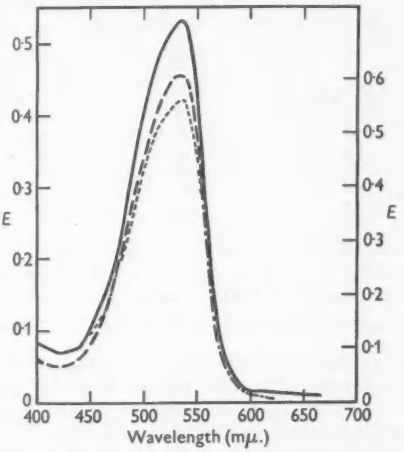

Fig. 3. Absorption spectra of zinc dithizonate solutions in CCl_4. ——, absorption spectrum of zinc dithizonate in CCl_4 (about 0·35 μg. Zn/ml.) — — —, ash solution from choroid (pH 4·75) extracted with dithizone in CCl_4. Absorption curve determined and corrected for excess dithizone (peak at 620 mμ.) - - - - -, ash from iris treated similarly. The curve for pure zinc dithizonate corresponds with the ordinates on the left, the other curves with those on the right.

(*d*) Copper solutions: $CuSO_4.5H_2O$ (0·1964 g.) was dissolved in 0·1 N-H_2SO_4 (1 l.). From this a solution containing 1 μg. Cu/ml. was prepared by dilution with 0·1 N-H_2SO_4.

(*e*) Zinc solutions: $ZnSO_4.7H_2O$ (0·2198 g.) was dissolved in 0·1 N-H_2SO_4 (1 l.). From this a solution containing 1 μg. Zn/ml. of 0·1 N-H_2SO_4 was prepared by dilution.

(*f*) Dithizone solutions (0·01 and 0·003 % w/v) were made up in A.R. CCl_4 and filtered. These solutions deteriorate slowly as a result of oxidation. Fresh solutions were made up every 3 months and stored in the dark at 0°.

Calibration. (i) *Copper:* solutions containing 0–10 μg. of metal in 10 ml. 0·1 N-H_2SO_4 were extracted with 0·003 % dithizone solution (6 ml.) in separating funnels. The lower layer was run off into a 10 ml. standard flask and the remaining drops washed through with CCl_4. Readings of the extinction at a wavelength of 510 mμ. were made on the spectrophotometer. A straight-line graph was produced by plotting the extinction against amount of Cu to be extracted. A similar graph was produced for a 0–25 μg. range by using proportionately greater quantities of reagents and final dilution of the CCl_4 extract.

(ii) *Zinc:* solutions containing 0–10 μg. of metal in 10 ml. 0·1 N-H_2SO_4 were placed in separating funnels and 5 ml. buffer (pH 4·75) and 1 ml. sodium thiosulphate solution were added. Each mixture was then shaken with successive 1 ml. portions of 0·01 % dithizone solutions until the lower layer (CCl_4) remained green after shaking for 2 min. Each portion was run off into a 20 ml. standard flask and the last drops were washed through with CCl_4. The solution was made up to volume and readings of extinctions at 535 and 620 mμ. were made.

A straight-line graph was produced by plotting $E_{535\,m\mu.} - 0{\cdot}25\ E_{620\,m\mu.}$ against the amount of Zn (0–10 μg.) to be extracted. A similar graph was obtained for the range 0–50 μg. Zn, after diluting the final extract to 50 ml.

Blank estimations were carried out with each set of Zn and Cu estimations as a precaution against casual contamination in the apparatus and standard solutions; these estimations also served as a check on the deterioration of the 0·003 % dithizone solution used to extract Cu. If the blank estimations were found to give a lower extinction than when the dithizone solution was fresh, the standard graph was recalibrated. The $E_{620\,m\mu.}/E_{535\,m\mu.}$ ratio of the 0·01 % dithizone solution was checked weekly, and the current value was used in calculating the absorption at 535 mμ. due to dithizone. The average value for the ratio was 4·0 and the greatest drop recorded in 3 months was from 4·2 to 3·8.

Specificity of the extraction procedures. Sandell (1944) states that several other metals are partially extracted, if present in certain relative concentrations, by the procedures used here for Cu and Zn. To determine whether such metals are present in amounts sufficient to cause significant interference in the present work, absorption curves for extracts from typical ash preparations of eye tissues were obtained by the above procedures.

In the case of Cu, all the dithizone shaken up with a solution of Cu salt in 0·1 N-H_2SO_4 is converted to copper dithizonate merely by the presence of a sufficient excess of Cu in the aqueous phase. Thus Fig. 2 compares the absorption curve of dithizone in CCl_4 shaken up with large excesses of $CuSO_4$ solution, and a solution of mixed eye-tissue ash, respectively.

In the case of Zn, excess dithizone is always present in the extract. However, if it is assumed that the absorption of a zinc dithizonate solution at 620 mμ. is zero (which is very nearly the case), then, if Zn is the only metal extracted, the absorption at 620 mμ. is solely due to dithizone. From the absorption curve for pure dithizone the relations between the $E_{620\,m\mu.}$ value and the E values of a dithizone solution at other wavelengths is known, and thus the amount of light absorption at any wavelength due to dithizone in a mixed solution of zinc dithizonate and dithizone, can be found. By subtraction of the E values calculated to be due to dithizone, from the total E values at various wavelengths of such a mixture of dithizone and zinc dithizonate, the extinction values which should be due to zinc dithizonate, if the extraction procedure is specific, can be found. The absorption curve, plotted from E values obtained in this way for an extract of a typical eye-tissue ash, is given in Fig. 3. There is an almost exact correspondence between this calculated curve and a pure zinc dithizonate curve, obtained by decomposing the excess dithizone with Na_2S solution, in an

extract from a solution of $ZnSO_4$ under the standard conditions described above. Such a correspondence would hardly occur if another metal dithizonate were present, even though the original assumption, that the 620 mμ. absorption was wholly due to dithizone, was unjustified.

The correspondence between the Cu curves demonstrates that here, also, the extraction procedure is specific for Cu.

The curves, both for Zn and Cu, agree with those given by Fischer & Weyl (1935) and Fischer (1937) for the pure metal dithizonates.

Estimation procedure. The solution of ash in 0·1 N-H_2SO_4, obtained as described in the section on preparation of tissues for analysis, was made up to a standard volume with 0·1 N-H_2SO_4 (the exact volume depending on the weight and kind of tissue analysed) and a sample taken for Cu extraction. The sample was made up to approximately 10 ml. with 0·1 N-H_2SO_4 and shaken with 6 ml. of 0·003% dithizone solution in a separating funnel. If the CCl_4 layer was then red, a further 9 ml. of dithizone solution were added and the whole again shaken. The extract, either 6 or 15 ml. in volume, was then run off and made up to volume as described before. Comparison of the value of the extinction at 510 mμ. with one of the standard graphs then indicated the amount of Cu present.

A smaller sample was taken for Zn estimation. This was first made up to approximately 10 ml. with 0·1 N-H_2SO_4 in a separating funnel and extracted with excess dithizone solution to remove Cu. Buffer (pH 4·75) and sodium thiosulphate solutions were then added and Zn extracted as described in the section on calibration. The extract was washed through with CCl_4 and made up to a volume putting the extinction values within the effective range of the spectrophotometer. The amount of Zn present was obtained by referring to the standard graphs.

Accuracy of the method. The reproducibility of the results was tested by homogenizing a mixture of cattle irises, choroids and lenses in a Waring Blendor. Portions of the homogenate were dried, reduced to ash and the Zn and Cu estimated by the standard procedure given above. Dry weights of the homogenate portions were of the order of 0·5 g., which was a rough average figure for the dry weights of the whole tissues taken for analysis in the following work.

Results

The results are summarized in Tables 1–4. In Table 1, samples 1–6, inclusive, were ashed in silica crucibles, and samples 7 and 8 in platinum crucibles. When tissues containing little organic matter, such as the aqueous and vitreous humours, were ashed, the deviation from the mean for samples in silica crucibles was somewhat greater than that shown above, and the mean of the results for these samples was consistently less than that for samples ashed in platinum crucibles. This effect is presumably due to

Table 1. *Reproducibility of zinc and copper estimations on eight equal portions of a homogenate of cattle lenses, irises and choroids*

Sample	Zinc (μg./g. dry material)	Copper (μg./g. dry material)
1	168	87
2	161	90
3	159	85
4	160	81
5	154	86
6	148	80
7*	170	92
8*	160	85
Mean	**160**	**86**
Standard deviation	7·03	3·80

* Ashed in Pt crucibles; all others in silica crucibles.

Table 2. *Concentration of zinc in the eye tissues of some mammals*

(A dash indicates that no analysis was made. Numbers in brackets denote μg./g. wet tissue.)

Tissue	Cattle (μg./g. dry tissue)	Sheep (μg./g. dry tissue)	Sperm whale (μg./g. dry tissue)	Rabbit	
				Coloured (μg./g. dry tissue)	Albino
Iris plus ciliary body	246 (41·0)	436 (65·1)	99·5	127	54·4
Choroid plus pigment epithelium	139 (26·5)	277 (69·2)	37·2	466	86·2
Retina minus pigment epithelium	71·0 (7·2)	80·0 (7·3)	54·1	—	—
Lens	37·3 (15·0)	117 (47·2)	35·2	15·8	12·5
Aqueous humour	30·0 (0·29)	—	—	—	—
Vitreous humour	26·4 (0·35)	23·2 (0·29)	10·5	—	—
Sclera	14·6 (4·1)	56·0 (16·1)	0·33	—	—
Cornea	13·5 (2·3)	25·0 (3·6)	35·3	6·6	12·1
Optic nerve	6·8 (2·2)	—	—	—	—

Table 3. *Concentrations of copper in the eye tissues of some mammals*

(A dash indicates that no analysis was made. Numbers in brackets denote μg./g. wet tissue.)

Tissue	Cattle (μg./g. dry tissue)	Sheep (μg./g. dry tissue)	Sperm whale (μg./g. dry tissue)	Rabbit (μg./g. dry tissue)	
				Coloured	Albino
Iris plus ciliary body	27·5 (4·6)	50·1 (7·4)	5·9	11·6	14·7
Choroid plus pigment epithelium	9·8 (1·7)	13·5 (3·4)	2·2	16·8	21·0
Retina minus pigment epithelium	6·8 (0·67)	11·5 (1·3)	10·6	—	—
Lens	1·2 (0·46)	2·1 (0·8)	4·2	0·62	0·49
Aqueous humour	10·4 (0·10)	—	—	—	—
Vitreous humour	17·7 (0·24)	24·0 (0·3)	5·9	—	—
Sclera	4·8 (0·13)	5·1 (1·4)	0·074	—	—
Cornea	3·2 (0·57)	1·9 (0·26)	3·4	0·30	1·5
Optic nerve	5·6 (1·8)	7·9 (2·6)	—	—	—

the formation of stable silicates. (Only two platinum crucibles were available, so that silica crucibles were used for all analyses except where otherwise stated.)

Table 4. *Zinc and copper concentrations in the eye tissues of three individual sheep*

(Results are in μg./g. dry tissue.)

Animal	Lens		Iris		Sclera	
	Cu	Zn	Cu	Zn	Cu	Zn
1	2·8	124	40	458	4·6	41
2	1·7	129	41	401	6·6	47
3	2·1	92	69	450	4·0	67

The results obtained for Cu in Table 1 are not indicative of the physiological Cu concentrations of the tissues used, for it has been observed that any aqueous solution placed in the Waring Blendor accumulates Cu, presumably from the alloys of the stirring mechanism.

Discussion

Table 2 shows that the zinc concentrations in the various types of tissue in mammalian eyes differ to a considerable extent. The differences are, in most cases, very much greater than could be accounted for by the experimental error (Table 1). The differences between the concentrations of copper in the various tissues, shown by the results of Table 3, are not so great, but in most cases are larger than the latitude, indicated in Table 1, which could arise from the experimental procedure. Table 4 shows that the differences in zinc and copper concentrations between the eyes of three individual sheep were small compared with the differences between the various types of tissue of the same sheep. Variation between individuals of the same species was not studied further because the eyes were obtained from a large abattoir and it was not easy to ascertain the breed, or the history, of the animals.

That the differences between the various eye tissues go deeper than peculiarities either of individuals or species, is indicated by the fact that the tissues can be placed in an order (in respect of their zinc and copper concentrations) which is roughly the same in each of the species examined. This order is very similar to that obtained from the results of Leiner & Leiner (1944) on the zinc content of fish-eye tissues, though the absolute concentrations were often much higher than those in the mammalian species examined here.

The results given in Table 2 for zinc concentrations in cattle-eye tissues are very much higher than any of the values found by Tauber & Krause (1943) for bullocks. However, the specificity of their method of estimation is questionable. They extracted the dithizone complex from an aqueous solution, the pH of which was probably acid, and in any case was never rigorously controlled. The reaction of dithizone with zinc can be made specific only in a solution at about pH 4·75, and in the presence of sodium thiosulphate or some other suitable complex-forming reagent. In acid solution, without any added reagents, only copper is likely to be extracted. The zinc/copper ratio found by them is actually never much greater than 1, whereas the zinc content of most biological materials is several times as great as that of copper.

The distribution of zinc and copper between the various types of sheep-eye tissues coincides with that found by Shakir (1948), and the concentrations of zinc found in each case are very similar. The concentrations of copper shown in Table 3 tend to be somewhat lower than the results for corresponding tissues given by Tauber & Krause for cattle, and by Shakir for sheep; this difference may be due to individual variation of the animals and to variation in pasture food.

It is significant that, both in the present work on zinc and copper in mammalian eyes and in the work on zinc in fish eyes, the highest concentrations of the metals were found in the pigmented parts of the eye. It is relevant that the only species (of those examined here) in which the retina, or any other tissue, contains a higher concentration of either metal than the iris, or choroid plus pigment epithelium, is the whale, whose choroid is thick, spongy and low in pigment. In addition, the whale eyes had been preserved and, on dissection, it was found impossible to separate spots of adhering pigment epithelium from the almost liquid retina.

The results for coloured and albino rabbit eyes show without doubt that the high concentration of zinc in the iris and choroid tissues is dependent, at least partly, on the presence of melanin pigment. No large difference was observed between the copper concentrations in the two types of eye, but the total amount of copper estimated was so small in each case (0·8–2·0 μg.) that the error introduced by ashing makes the results probably not significant.

Flesch (1949) reported a connexion between the concentration of copper in rabbit hair, and pigmentation. He observed a small difference between the copper concentrations in white and black hair from the same rabbit, though white hair from one rabbit might contain more copper than the black hair from another. Fore (1950) found no difference in manganese contents of the two types of hair, but a big drop in ash weight in white hair. We have found a small lowering of zinc concentration in white hair, no difference in copper concentration, and again a big drop in ash weight. Thus, in the rabbit, it appears that though zinc and copper may be concerned in the pigment problem, some other mineral element or elements are present in enhanced quantity in pigmented hair.

2. FRACTIONATION OF PIGMENTED TISSUES

Flesch (1949) has shown that pigment separated from a mouse melanoma contains much more copper than the melanoma tissue itself. Leiner & Leiner (1944) showed that grey or black powders, separated by differential centrifugation from powdered dry fish eyes, contained rather more zinc than colourless fractions, though a yellow fraction contained more still. They did not postulate a connexion between zinc concentration and pigmentation.

In view of these facts and the evidence recorded in the first part of this paper, it was decided to determine whether zinc and copper are concentrated in the pigment fraction of the iris and choroid tissues of mammals.

Experimental methods and results

It was established by analysis that cattle irises and choroids from left and right eyes of the same animal differ in their zinc concentrations by less than 15% of the mean between the two, and in their copper concentrations by less than 20% of the mean.

A number of pairs of cattle eyes were obtained from the abattoir; one of each pair of irises dissected out was analysed whole, and the other of the pair fractionated to obtain pigmented material.

Fractionation

Whole irises (3–6) were digested with 5 ml. of an approximately 2% (w/v) trypsin suspension and about 25 ml. of twice-distilled water for 24–36 hr. at 37°. The melanin pigment can then almost all be washed away from the bulky residue of undigested fibrous, connective and muscular tissue, which is allowed to remain in the digestion flask.

Table 5. *Zinc and copper present in various fractions of cattle irises obtained by digestion with trypsin*

Fraction	Dry weight (g.)	Zinc (μg.)	Copper (μg.)
Exp. 1			
Undigested residue	0·0430	9·6	0·9
Combined washings	0·2679	24·6	8·0
Pigment fraction	0·1383	101·0	9·0
Total	0·4462	135·2	17·9
Trypsin blank	0·0508	23·6	1·9
Whole irises ashed	0·5050	99·0	9·8
Total	0·5558	122·6	11·7
Exp. 2			
Undigested residue	0·2175	22·8	2·4
Combined washings	0·1945	11·1	4·6
Pigment fraction	0·0997	74·5	8·0
Total	0·5127	108·4	15·0
Trypsin blank	0·0534	23·4	2·4
Whole irises ashed	0·4992	79·6	9·8
Total	0·5526	103·0	12·2
Exp. 3 (calves)			
Undigested residue	0·1766	9·0	4·0
Combined washings	0·2048	17·8	4·2
Pigment fraction	0·1207	62·8	5·3
Total	0·5021	89·6	13·5
Trypsin blank	0·0431	12·0	2·8
Whole irises ashed	0·4647	80·0	10·5
Total	0·5078	92·0	13·3

The washings were centrifuged until the supernatant liquid was yellow, and this solution was then poured off. The residue was stirred up with twice-distilled water and

recentrifuged, the operation being repeated five or six times, until no opacity due to protein was observable in the washings. The washings were all added to the original supernatant liquid.

The undigested residue, the washings, the pigment fraction and a blank containing 5 ml. of the trypsin suspension were all dried, ashed and analysed for Zn and Cu by the methods already described. The results are compared in Table 5 with the analysis for the 'control' irises, which had been dried and ashed whole. These results, plus the trypsin blank figures, should equal the total for the various fractions if no contamination or loss has occurred.

Pairs of whale eyes were not available, and digestion of the irises, possibly due to the presence of fat, only partially separated the pigment from the rest of the tissue. Thus it was not possible to construct a balance sheet showing the amounts of Zn and Cu to be found in the pigment fraction and in the other parts of the tissue. However, in the pigment which was separated, Zn and Cu were present in much greater concentration than in the original tissue.

It was likewise found impossible, even after 3 or 4 days digestion, to separate more than a small amount of the pigment from cattle choroids, in the manner used for irises. It was again found, however, that the pigment fraction which was isolated was much richer in Cu and Zn than the original tissue. In Table 6 are collected the results of analyses of dialysed pigment fractions from the various sources mentioned above.

Table 6. *Zinc and copper concentrations in various pigment samples, prepared from eye tissues by the trypsin digestion procedure followed by dialysis*

Source of pigment	Zinc (μg./g. dry material)	Copper (μg./g. dry material)
Adult cattle irises:		
Batch 1	1000	78
2	730	65
3	719	87
4	748	80
Calf irises	522	44
Humpback whale irises	968	56
Adult cattle choroids	737	48

Further examination of the pigment fraction

Renewed digestion. Part of the pigment fraction obtained from six cattle irises by the above procedure was analysed as such for Zn and Cu, and part was digested with 2% (w/v) trypsin suspension for a further 3 days at 37° before analysis. Results for the two analyses are shown in Table 7.

Table 7. *Zinc and copper concentrations in two pigment preparations before and after a second trypsin digestion*

Pigment preparation	Zinc (μg./g. dry material)		Copper (μg./g. dry material)	
	Before	After	Before	After
1	871	1073	107	84·9
2	874	1028	113	82·1

Hydrolysis with acid. Part of a pigment fraction was analysed intact, and part weighed and then treated with 0·1 N-HCl for 1 hr. on a steam bath. The insoluble residue was centrifuged down, washed, dried, weighed and analysed. Results are shown in Table 8.

Table 8. *Effect of acid hydrolysis upon the zinc and copper concentrations and the dry weight of insoluble material, of an iris pigment fraction*

	Dry weight (g.)	Zinc content (μg.)	Copper content (μg.)
Original pigment material (*A*)	0·1225	100	9·1
Insoluble residue after hydrolysis of (*A*)	0·0741	0·8	8·1

Part of the insoluble residue was retained as a wet suspension. The absorption curves of this suspension in twice-distilled water and of a suspension of the original pigment fraction are given in Fig. 4.

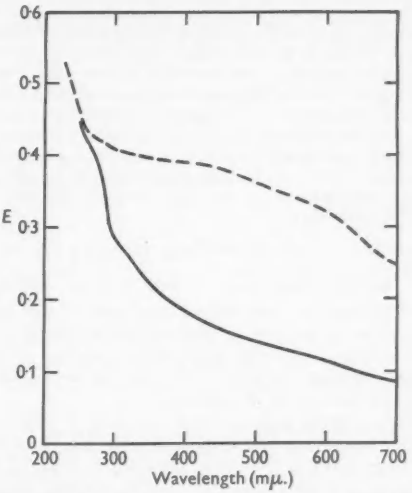

Fig. 4. Absorption spectra of suspensions of melanin material from cattle irises, before and after acid hydrolysis. ——, absorption spectrum of the original melanin material suspended in twice-distilled water (pH 5·0). — — —, absorption spectrum of a suspension of the insoluble residue after hydrolysing melanin material.

The amount and nature of the ash of the pigment fraction. The percentage of the dry weight of the pigment fraction recoverable as ash was determined for a sample repeatedly washed and centrifuged down from twice-distilled water, and on a sample dialysed for 3 days against twice-distilled water. Ashing was carried out in a platinum crucible and the respective percentages for the two samples were found to be 2·594 and 2·590.

It is clear that no more than a small part of the ash can be accounted for by Zn and Cu salts or oxides, for these make up only 0·077 and 0·0063%, respectively, of the dry weight of the pigment sample.

It was therefore decided to investigate the other metallic constituents of the ash by a qualitative emission spectroscopic technique.

The Hilger E 1·301 version of the Littrow emission spectrograph was used, and an arc source was used for excitation of the electrode and ash.

By means of the Hartmann diaphragm three spectrograms were taken without moving the photographic plate: (*a*) a control consisting of pure graphite rods; (*b*) the test material placed on a hollowed graphite cathode opposite a graphite anode and (*c*) a reference standard consisting of two pure iron electrodes.

Spectrograms were photographed for two wavelength ranges: 2250–2880 and 2880–5050 A.

The two spectrograms for these ranges, each comprising three spectra in exact juxtaposition, were examined on a Judd Lewis comparator. Lines in the test spectrogram not present in the control were identified by reference to the iron lines in the third spectrogram. The iron spectrogram was charted, and the unknown lines in the test spectrogram identified by reference to labelled photographs in Brode (1939) and the wavelength and element tables in the Massachusetts Institute of Technology *Wavelength Tables* (1939). Unequivocal 'raies ultimes' were identified for Ca, Ba, Mg, Fe, Cu and Zn. The number of iron lines in the test spectrogram was very large and it appears that this element was the major metallic constituent of the ash. These metals, together with Na, were identified in a similar manner in a batch of irises which had been repeatedly washed with twice-distilled water. This demonstrates that they have not been introduced into the material during the digestion or dialysis procedures.

Discussion

The results presented in Table 5 show, without further inquiry, that greater amounts of zinc and copper are associated with the pigment fraction of cattle irises than with any other fraction. However, on close inspection of the results, several matters are seen to require comment.

It was to be expected that the sum of amounts found in the iris fractions would be less than the amount in whole iris since losses due to decomposition of organic compounds are inevitable during digestion and in evaporation at 100° of the large quantities of water present in the washings. Differences between the dry weights and zinc and copper contents of the two irises from the eyes of the same animals are also bound to occur as a result of biological variation and imperfect separation in dissection. Taking these factors into account, there appears to be fairly good agreement between the results for corresponding groups of irises, and it may be concluded that little or no contamination or loss of zinc and copper took place.

The proportion of the total dry weight, zinc content, and copper content, found in each fraction varied somewhat from one experiment to another; this was probably due to variations in the extent of digestion of the tissue and to small variations in the separation technique.

In each experiment, the concentrations of zinc and copper in the dry pigment were much higher than in the other fractions of the original tissue. The accumulation of zinc was much greater than that of copper, and the concentration of zinc was increased by further trypsin digestion of the pigment fraction, whereas that of copper was decreased. Dialysed samples of pigment retained the high concentrations of both metals. Pigment fractions isolated from cattle choroids and from humpback whale irises contained concentrations of zinc and copper similar to those in cattle iris pigment fractions.

Table 5 shows that the high concentrations of zinc and copper in cattle irises can be ascribed to accumulation in the pigment fraction. Table 6 shows that similar accumulations occur in cattle choroids and whale irises. In conjunction with the results in the first part of this paper, it thus appears that the pigment material in mammalian eyes is closely associated with much more zinc and copper than is present in most mammalian tissue constituents.

That the pigment from cattle irises is of the melanin type, is indicated by the retention of colour in the portion of the pigment fraction which is insoluble and resistant to acid hydrolysis. Natural melanins have usually been isolated as the insoluble residues of prolonged acid or alkaline hydrolysis of pigmented tissues. The term 'melanin' is ill-defined; different workers have used different combinations of properties to characterize the material, and it is by no means certain that all the substances which have been given this name have the same chemical structure. The tests for melanin identity employed in the present work are quoted in the classification by Mason (1948).

Physical evidence for the identity of the pigment from cattle irises is given by the absorption curves (shown in Fig. 4) for suspensions of the original pigment fraction, and of the residue, after acid hydrolysis of this fraction. These are similar to those for melanins from other sources (Edwards & Duntley, 1939; Serra, 1945; Zwicky & Almasy, 1935). Shakir (1948) obtained similar curves for pigment in a supernatant suspension made by grinding choroids; this material had a higher concentration of zinc and copper than the whole tissue. A chemical test used by many workers (e.g. Sachs, 1944) for melanin is the bleaching of the colour by strong oxidizing agents. Concentrated nitric acid rapidly destroyed the colour of the material obtained by us.

A material analogous to the pigment fraction of cattle irises was obtained by Greenstein, Turner & Jenrette (1940), who showed that the black insoluble fraction from mouse melanomas hydrolysed with trypsin contained much protein. This protein

dissolved on acid hydrolysis, leaving a highly coloured residue having a nitrogen content which coincided with that of melanins synthesized from tyrosine.

It is considered that the evidence presented gives sufficient justification for placing the chromogenic material of the pigment fraction from cattle irises in the melanin category.

It has now been fairly well established that mouse-melanoma tissue contains a tyrosinase and that copper is an essential part of this enzyme (Lerner, Fitzpatrick, Summerson & Calkins, 1950). Flesch (1949) suggests that copper becomes bound to the pigment in the neighbourhood of the sulphur atoms of sulphur-containing amino-acids of the attached protein. The relatively large amount of copper in the pigment fraction certainly cannot be due to tyrosinase in the free state, for the enzyme is water-soluble, and would have been removed in the fractionation process.

The results given in Tables 4 and 5 show a difference between the states of zinc and of copper in the pigment fraction. The concentration of copper was slightly reduced by both trypsin digestion and acid hydrolysis. The concentration of zinc was slightly increased by trypsin digestion and reduced almost to zero by acid hydrolysis. This indicates a probable difference between the binding of the two metals in the pigment fraction.

The results of the emission spectroscopic analysis show that the accumulation of metals is not limited to zinc and copper. These two metals together constitute only 0·083 % of the dry weight and 3·21 % of the ash of the pigment fraction. The iron, calcium, magnesium and barium, which were the only other elements identified, together must therefore account for much more of the ash weight than do zinc and copper. The great number and strength of the iron lines in the spectrum indicate that this is a major constituent of the ash.

The demonstration of the presence of barium confirms the report of Ramage & Sheldon (1931) that this metal occurs in irises and choroids of cattle, and in the pigment separated from these tissues by rubbing. They did not find the metal in any other cattle tissues, nor in the choroids of a number of other animals. Waelsch (1932) found that melanin from the choroid gave an ash which was 1·9 % by weight of the dry material; this ash contained a demonstrable concentration of iron.

It thus appears that a number of metals are accumulated in the pigment fraction of mammalian eyes in unusually high concentration. This circumstance may perhaps be connected with a number of other observations. As mentioned in the first part of this paper, white rabbit hair contains much less ash material than black. Gortner (1911) showed that black pigment from rabbit hair, horse hair and from black feathers contained 2–3 % ash, which was chiefly iron oxide. Rothman & Flesch (1943) isolated a red pigment, containing iron in the ferric form, from bright-red human hair. They stated, however, that such a pigment had not been obtained from any human hair except red, nor from the hair of any other animals. Giuliani (1938) found that dried ink from the squid (*Sepia officinalis*) gave a total ash of 1·86 % of the dry weight, and a copper analysis of 1·17 % of the dry weight.

There exists no direct evidence yet which might settle the problem of whether the high concentrations of these metals in pigmented tissues, and in pigment fractions of these tissues, have any function in developing or maintaining the natural coloration.

SUMMARY

1. Details of modified standard microprocedures for estimating copper and zinc are presented. The methods, as applied to eye tissue, are shown to be specific for these metals.

2. Results are presented for copper and zinc concentrations in eye tissues of sheep, cattle, whales, and rabbits. It is shown that differences in the concentrations of zinc and copper between the various tissues exceed the experimental error.

3. Ranged in respect of zinc and copper concentrations, the tissues fall in roughly the same order in each of the species examined. The highest concentrations, in general, are in the pigmented tissues.

4. The zinc content of the iris or choroid of albino-rabbit eyes is lower than that of the same tissues of pigmented rabbits. There is no corresponding difference in zinc content for the lens or cornea.

5. The zinc and copper of irises occurs mainly in pigment material found in the supernatant liquor from a trypsin digest of the tissue. The concentrations of zinc and copper are higher in the pigment material than the original tissue, and are not reduced by dialysis.

6. The zinc concentration in the insoluble pigment material is slightly increased by prolonged trypsin digestion, but reduced almost to zero by acid hydrolysis. The copper concentration is slightly reduced by trypsin digestion and little affected by acid hydrolysis.

7. The ash of dialysed pigment fractions contains calcium, magnesium, barium and iron, in addition to zinc and copper. Iron appears to be the major constituent.

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Distribution of Copper and Zinc in the Eyes of Fresh-water Fishes and Frogs. Occurrence of Metals in Melanin Fractions from Eye Tissues

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The results of Leiner & Leiner (1944) showed zinc to be present in the choroid, and other pigmented tissues, of the eyes of fresh-water fishes from Lake Constance, in amounts as much as one hundred times those in similar tissues from mammalian eyes (figures for which are given by Bowness, Morton, Shakir & Stubbs, 1952). Very high concentrations of zinc in the pigmented eye tissues were reported by Leiner & Leiner for each of nine species of fish from Lake Constance; indeed, many were higher than any previously reported to occur normally in living matter (cf. Monier-Williams, 1949). The highest recorded concentration of zinc in animal products, prior to the work of Leiner & Leiner (1944), was for serpent venom, namely 0·56 % of the dry weight (Delezenne, 1919). Monier-Williams (1949) attributes even higher concentrations to tench and herrings (Bertrand & Vladesco, 1921). The original paper does not in fact record very high values, and the mistake arose presumably from an erroneous abstract (*Analyst* (1921), **46**, 244). The range of zinc concentrations found by Leiner & Leiner in the dry choroids of fresh-water fishes was from 0·44 to 2·96 %, and the average for all the species examined was 1·18 %.

It was possible that the accumulation of zinc might be a peculiarity arising only in Lake Constance, or alternatively, that very high concentrations of zinc in some eye tissues are common to fresh-water fishes everywhere.

The present investigation had two main objects: (i) to ascertain whether the results obtained by Leiner & Leiner can be confirmed by studies on fishes from Lake Windermere, and (ii) to determine whether the association between zinc concentration and melanin pigmentation found in mammalian eyes (Bowness *et al.* 1952) can be demonstrated for fresh-water fishes. Leiner & Leiner (1944) fractionated a powdered dry homogenate of whole fish eyes by differential centrifugation in an attempt to discover the nature of the material to which most of the zinc was bound. They found that black and grey fractions contained most of the zinc, but, as these accounted for the greater part of the original material, they did not achieve any significant increase in the concentration of zinc, and were unable to indicate the nature of the material to which the metal was bound. In the present work, the trypsin digestion technique (Bowness *et al.* 1952), by which a pigment fraction was separated from mammalian

irises, was found to be effective in separating an analogous black material, rich in zinc, from fish and frog choroids.

MATERIALS AND METHODS

Materials. The heads of trout (*Salmo trutta*, Regan) and whole perch (*Perca fluviatilis*, C.) were obtained through the courtesy of the Freshwater Biological Association at Lake Windermere. The fish were despatched packed in cloth, surrounded by ice in fish tins; the eyes were removed from the heads and dissected at Liverpool within 2 days of the death of the animals. The first batch of perch obtained were young and small, the second batch varied from small to fully grown and aged fish.

Edible frogs (*Rana esculenta*) were obtained alive from Holland through Nederlandsche Heidemaatschappij, Arnhem. The animals were guillotined and the eyes dissected within, at most, a few hours; the retinas were utilized for other work in this Department.

English frogs (*R. temporaria*) were obtained alive from Mr H. F. Ashton of Norwich. The animals were chloroformed, and the eyes dissected, at most within a few hours.

The size of both types of frog varied, but most of them were probably hatched during the previous season.

Dissection of eyes. Certain differences between fish eyes and mammalian eyes should be noted here. In many fish, the inside of the sclera is covered by the silver membrane (argentea). This contains crystals of guanine which produce a silvery-white reflecting surface. In the posterior portion of the eye, between the silver membrane and the choroid, are pockets of blood, which, when the eyes come to be dissected, has clotted. These pockets are associated with the choroid body or 'gland'.

The technique of dissection used was similar to that described by Bowness *et al.* for mammalian eyes. The retinas from fish or frog eyes could be detached without adhering pigment by pulling the optic nerve while exerting a gentle pressure on the eyeball. The retina could then be carefully drawn out of the eye, attached to the optic nerve. With fish eyes, after the separation of the choroid from the blood clot, small pieces of silver membrane were found still to adhere to the tissue; these could only be removed by teasing the choroid with tweezers and a small scalpel. The irises of the fish were never completely separated from argentea.

Analyses for copper and zinc. The eye tissues from the frog *R. temporaria* and from perch were very small; after washing them with twice-distilled water, it was not found practicable to remove the excess water with filter paper. Most of the concentrations are therefore expressed only on a dry-weight basis, wet-weight concentrations having been obtained only for trout and some of the larger tissues of *R. esculenta*.

The analytical method has already been described (Bowness *et al.* 1952). The accuracy of the method as applied to fish-eye tissues has not been assessed experimentally, but it should not be less than that obtained with mammalian material. The ratio of the amount of Zn to a given weight of organic material is much greater for fish eyes than for mammalian eyes; the error in estimating Zn concentration which is due to loss in the course of ashing the organic material is therefore much less important for fish tissues. The generally smaller size of the fish eyes, and the consequent increased greater difficulty of dissection, resulted, however, in increased uncertainty due to adhering extraneous tissue. It is therefore difficult to assess the extent of uncertainty arising from experimental procedure for the figures given in Tables 1 and 2; in general, it is mainly the third figure which is doubtful.

Separation of pigment fractions. Initially, an attempt was made to separate a pigment fraction from irises, as was done for cattle. However, it was found impossible to remove all the adhering silver membrane. The crystals of guanine accompanied the pigment in the fractionation and could not be removed without the use of hydrolysing agents (e.g. HCl), which are liable to split off the metals from the pigment material.

Attention was then transferred to the choroid. By careful dissection, this could be completely freed from guanine-containing material. It was found to be softer and less fibrous than the choroids of cattle and sheep, and therefore might be expected to be more readily digested by trypsin. Choroid tissue from perch and frogs was, in fact, split up entirely in 3–7 days by digestion with a pancreatin and trypsin suspension, but the process was not successful for trout. At the end of the time required for the choroids to be disintegrated, the digest was filtered through glass wool to remove any undigested tissue, and the filtrate centrifuged until no pigment remained in suspension. The supernatant was poured off, and the black residue stirred up with twice-distilled water and recentrifuged. This procedure was repeated four times. The final residue is the pigment-protein fraction.

Emission spectroscopic technique. Emission spectra of the ash of the perch melanin-protein fraction were recorded by the use of a carbon arc and a high-dispersion Littrow spectrograph (Hilger E. 1·301). Details of the technique used for identifying the elements present are given by Bowness *et al.* (1952).

RESULTS

The main findings are displayed in Tables 1 and 2. The recorded results have been calculated directly from the experimental readings.

In order to test whether high concentrations of zinc, such as those in some of the eye tissues, are of general occurrence in the body of the perch, analyses were carried out on liver, muscle and skin.

Proportion of the copper and zinc in the perch choroid bound to the insoluble pigment-protein fraction. The dry matter in 73·3 mg. of fresh perch choroid was found to weigh 8·6 mg. When 309·2 mg. of fresh choroids, corresponding to 36·2 mg. dry weight, were digested with trypsin and a pigment-protein fraction isolated from the supernatant, the dry weight of this fraction was 14·9 mg. The weights of zinc and copper present in these amounts of choroid and pigment-protein material can be calculated by using the appropriate concentrations of the metals given in Tables 1 and 2. The ratio of the weight of zinc or copper in 14·9 mg. pigment-protein to that in 36·2 mg. choroid shows the proportion of the total weight of these metals in the choroid which is in non-ionic combination with the pigment-protein material. For zinc, this proportion

Table 1. *The concentrations of copper in the eye tissues of trout, perch and frogs*

(The ordinary figures show the concentrations in μg./g. dry tissue; the figures in brackets underneath (when given) show the concentration in μg./g. wet tissues corresponding to the figures immediately above. A dash means that the tissue was absent, or not available for analysis.)

Tissue	Trout (fresh-water)	Perch	Frog	
			R. temporaria	*R. esculenta*
Lens	5·62 (2·42)	0·85	29·8	27·4 (13·6)
Cornea	18·4 (3·5)	21·0	104	—
Sclera	8·65 (2·05)	27·3	120	199 (8·87)
Retina	35·8 (4·85)	18·1	117	—
Argentea	199·0	17·1	—	—
Iris plus ciliary body	105·0 (26·5)	18·8	—	—
Choroid	87·8 (18·0)	30·5	133	—
Pigment-protein fraction of choroids	—	49·3	—	985

Table 2. *The concentrations of zinc in the eye tissues of trout, perch and frogs*

(The ordinary figures show the concentrations expressed as μg./g. dry tissue; the figures in brackets underneath, when given, show the concentrations in μg./g. wet tissue corresponding to the figures immediately above.)

Tissue	Trout (fresh-water)	Perch	Frog	
			R. temporaria	*R. esculenta*
Lens	15·7 (6·75)	15·6	78·5	16·3 (8·1)
Cornea	81·3 (11·5)	189	278	—
Sclera	457 (95·6)	343	326	266 (132)
Retina	580 (74·2)	728	500	—
Argentea	7020	2010	—	—
Iris plus ciliary body	5990 (1560)	6380	—	—
Choroid	15100 (3100)	23800	14400	—
Pigment-protein fraction of choroids	—	43800	—	28100

is $\frac{43800}{23800} \times \frac{14{\cdot}9}{36{\cdot}2} \times 100 = 74{\cdot}7\,\%$ (see Table 2); for copper, $\frac{49{\cdot}3}{30{\cdot}5} \times \frac{14{\cdot}9}{36{\cdot}2} \times 100 = 53{\cdot}0\,\%$ (see Table 1). The pigment-protein material, to which these fractions of the total copper or zinc are bound, constitutes 41·6 % of the dry weight, or 4·82 % of the wet weight of the choroid.

Table 3. *Concentrations of zinc in perch skin, muscle and liver*

Tissue	Zinc concentration (μg./g. dry tissue)
Skin (white)	31·0
Muscle	57·7
Liver	59·6

Absorption spectra of pigments. Light passing through a suspension of particles in water is partly scattered and partly absorbed by the particles. The scattering of light by a particle depends on its size. The absorption curve of a suspension will, therefore, be governed by two factors, the light absorption of the substance suspended (itself dependent on the structure of the compound), and the size and number of particles suspended.

The absorption curves of suspensions in water of melanin-protein fractions from perch and frogs are given in Fig. 1.

Hydrolysis of pigment-protein fraction. Prolonged trypsin digestion of melanin-protein fractions from perch and frogs was found gradually to lower their zinc and copper contents. The zinc content on dry

weight of the perch material was reduced from 4·38 to 4·1% by 3 days' incubation with trypsin suspension. It is possible, therefore, that the original figure of 4·38% is too low by virtue of the incubation with trypsin during the separation procedure.

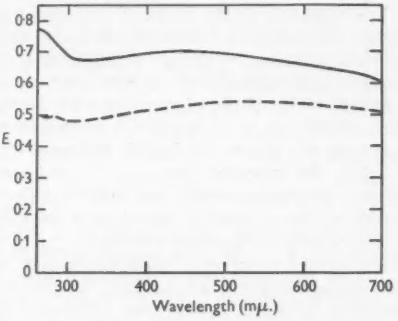

Fig. 1. Absorption spectra of melanin fractions from fish and frog eyes. ——, perch pigment; – – –, frog (*R. esculenta*) pigment.

Acid hydrolysis of the pigment was found to have an effect analogous to that obtained with cattle pigment. Part of the dry melanin-protein fraction (8·3 mg.) from perch was treated with 10 ml. 0·1 N-H_2SO_4 for 1 hr. at 100°. The resulting mixture was centrifuged, the residue being washed with twice-distilled water and recentrifuged twice. The final residue was dried, weighed, ashed and analysed for zinc.

The dry weight of the residue was 4·1 mg. and the zinc content 2·4 μg. The estimated zinc content of the original melanin-protein fraction would be 365·2 μg.

Acid hydrolysis has thus removed nearly all the zinc and half the dry weight of the original material.

Ash content of the melanin-protein fraction. The amount of dry material remaining as ash after incineration was found to be just over 10% for the whole choroid, and just under 10% for the pigment-protein fraction from perch. The ash from the choroid will include much dialysable material, so that there is evidently a higher concentration of bound metals in the pigment-protein fraction than in the original choroid.

Emission spectroscopic analysis of the ash from perch material showed that the main metallic constituents of the pigment-protein fraction are zinc, calcium, and iron. Minor constituents were aluminium, copper, magnesium and strontium. No trace was found of barium, which was identified in the ash of cattle pigment-protein fraction.

DISCUSSION

It is evident from Table 4 that the high concentrations of zinc obtained by Leiner & Leiner (1944) are not due to Lake Constance as a special habitat.

In all seven of the other species of fish from Lake Constance examined by Leiner & Leiner, the iris, choroid, and silver membrane had the highest zinc contents of any of the eye tissues. Of these three, the choroid usually had the highest concentration, but in the Burbot the iris was higher in zinc; in no case was the silver membrane highest.

In comparing the results for fish from Lake Constance and Lake Windermere there are several points to note. Leiner & Leiner give the name of their trout as 'Sea-trout' (*Trutta lacustris* L.). There is some dispute as to whether the trout all belong to the same genus, but in the present work the view of Regan (1911), who classified all the English trout as *Salmo trutta*, is followed. There must remain some doubt, however, as to whether both sets of fish are of the same species. Secondly, in considering the silver membrane, it should be noted that the tissue is very small and it is not possible to separate it entirely from pigmented material. The amount of pigment remaining in attachment is partly a matter of chance, and there is therefore much latitude for variation in the results. Thirdly, the choroid body was not analysed in the present work because it appeared to be mainly composed of blood; it was completely dissected away from the choroid and silver membrane and thus does not enter into any of the present results.

Table 4. *A comparison of the results of Leiner & Leiner* (1944) *for perch and trout eye tissues with those obtained in the present work*

Tissue	Trout (μg. Zn/g. dry tissue)		Perch (μg. Zn/g. dry tissue)	
	Leiner & Leiner	Present work	Leiner & Leiner	Present work
Lens	16·0	15·7	20	15·6
Retina	540–990	580	1090–1780	728
Iris plus ciliary body	6960–8700	5990	6200–7710	6380
Silver membrane	6360	7020	8200	2010
Choroid body	980–1490	—	510–1730	—
Choroid	9200–11350	15100	24000–29600	23800

The zinc concentrations in the eye tissues of the frog fairly closely parallel those of the fresh-water fish. Leiner & Leiner (1942) studied the concentrations of zinc in the eye tissues of many salt-water Teleosts. The general range of zinc concentrations was lower than that in fresh-water fish and frogs, but higher than that in mammals; for example, the range of zinc concentrations for the dry choroids of all the species of salt-water fish studied was 530–9800 μg./g. (Leiner & Leiner, 1944). In all the fish examined, whatever the absolute amounts of zinc present, the same tissues contain the highest concentrations of zinc. Zinc concentrations in the eye tissues decrease in the following order: choroid or iris, retina, sclera, cornea, lens. The argentea concentrations are rather variable. Excluding this last-named tissue, which does not occur in mammals, and excluding the mammalian lens, the same order is true for results obtained on cattle, sheep, whale and rabbit eyes (Bowness *et al.* 1952). The range, from highest to lowest zinc concentration, is very much smaller in mammals than in fish. The concentration in the lens of mammalian eyes is generally slightly higher than that of fish eyes, yet the concentration in the choroid may be only 1/100th that of the same fish tissue.

Table 3 shows that, despite the high concentrations of zinc in the eye tissues, other parts of the perch have much the same concentrations as those given by Lutz (1926) for various mammalian species.

The results for copper concentrations in various eye tissues do not follow any clear-cut general pattern. The variation in the order of eye tissues, arranged according to copper concentrations, is much greater than is the case for zinc. The physicochemical and biochemical agencies which accumulate zinc appear, in all the vertebrate eyes examined, to be active in one tissue more than another, and most active in the same tissues in all species. The same cannot be true for copper. In general, pigmented tissues and pigment-protein fractions from all the vertebrate eyes examined by us contain relatively high copper concentrations. In some species, other tissues may have concentrations as high, or higher. In trout, the silver membrane has a higher copper content than either choroid or iris. In connexion with this, it was observed that the debris from an attempted digestion of mixed choroids and silver membranes of trout, which consisted largely of guanine, had a high copper content (672 μg./g. dry tissue). Frog-eye tissues seem to have a relatively high copper content, the pigment-protein fraction of *R. esculenta* being especially high in copper.

The very high concentrations of zinc in the pigment-protein fractions of perch and frogs, and the observation that at least 74% of all the zinc in perch choroids is bound in non-ionic form to this fraction, show that the high zinc contents of the pigmented eye tissues can largely be accounted for by an association with melanin pigmentation.

As compared with up to 2·6% found in material from cattle, the ash content of dry perch pigment-protein fraction was about 10%, of which calcium and iron were shown to be fairly abundant constituents. Zinc, however, constituted nearly half of this ash, as compared with only 3·21% of the cattle material. Iron appeared to be the major metallic constituent of the cattle pigment-protein ash, and barium, which was not found in the perch material, was present as a minor constituent. It seems, therefore, that the absolute amount of the metallic elements associated with the pigment-protein material, and the relative proportions of each, vary from one vertebrate species to another.

The absorption curves of pigment-protein fractions from perch and frog eyes differ from those obtained from cattle (Bowness *et al.* 1952) and from those of other melanins (Edwards & Duntley, 1939; Serra, 1945; Zwicky & Almasy, 1935). The ratio of the light absorption in the ultraviolet region to that at higher wavelengths is much lower for the fish material than for the others. This may be caused partly by the different size of the particles in suspension, but such a large difference probably also indicates a difference in chemical structure.

Though it appears established that there is a connexion between melanin pigmentation and the presence of high concentrations of zinc (and possibly other metals) in the pigmented eye tissues, and perhaps in other pigmented tissues (cf. Bowness *et al.* 1952), the function of such metallic accumulations remains an open question. Leiner & Leiner (1941) showed that the zinc contents of the tissues of fish and mammals could not be accounted for solely by the amount of metal bound to carbonic anhydrase. They showed that zinc could lessen the inhibition of carbonic anhydrase by various tissue constituents. There seems no reason, however, why such a function should require a great accumulation of zinc in the pigmented eye tissues of fish, and especially in fresh-water fishes.

As stated previously (Bowness *et al.* 1952), the accumulation of copper in pigment fractions cannot be explained by the presence of free tyrosinase. The only other organic copper compounds known to occur in vertebrates are haemocuprein and hepatocuprein (Keilin & Mann, 1938), but these also are relatively water-soluble and could not account for the copper in the pigment fraction unless they were combined in some insoluble form.

Possible functions of the metals associated with the melanin-protein fractions of pigmented tissues will be discussed in a further paper.

SUMMARY

1. Results for the copper and zinc contents of eye tissues of perch, trout, and frogs are presented. The results of Leiner & Leiner (1944) for zinc in perch and trout are confirmed.

2. Most of the zinc, and half the copper, of the perch choroids is bound in non-ionic form to the pigment-protein fraction of the tissue.

3. The absorption spectra of pigment-protein suspensions from perch and frog eyes differ from those of cattle eyes.

4. On incineration, the perch pigment-fraction was found to leave about 10% of the dry weight as ash. Calcium, iron and strontium were constituents of this ash, but almost half is zinc (calculated as metal).

5. The association between zinc and melanin pigmentation is discussed.

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Effects of High-voltage Cathode Rays on Aqueous Solutions of Tryptophan, Tyrosine, Phenylalanine and Cystine

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The effects of irradiation by high-voltage cathode rays on amino-acids in fish muscle and on aqueous solutions of histidine monohydrochloride have already been reported (Proctor & Bhatia, 1950; Bhatia & Proctor, 1951). The irradiation caused no significant destruction of any one of ten amino-acids in fish. It was shown, however, that histidine in aqueous solution is decomposed by irradiation with cathode rays through deamination and fission of the glyoxaline ring.

Stenström & Lohmann (1928) concluded from their experiments on X-ray irradiation of aqueous solutions of tyrosine, that the phenolic structure of this amino-acid is changed by the irradiation. They also irradiated solutions of cystine and noticed no measurable change when the colorimetric method of Folin & Looney (1922) was used to determine cystine. Stenström & Lohmann (1931) also reported that ammonia was given off from tyrosine and that tryptophan was destroyed upon irradiation with roentgen radiations. Buckhman & Manoilov (1949) found that cysteine evolves hydrogen sulphide on irradiation with ultraviolet rays. Dale & Davies (1951) studied the liberation of hydrogen sulphide by X-radiation from aqueous solutions of cysteine and glutathione.

The present study was undertaken to ascertain the effects of various doses of cathode rays on aqueous solutions of DL-tryptophan, DL-phenylalanine, L-tyrosine and L-cystine in various concentrations.

EXPERIMENTAL

Stock solutions of the amino-acids were prepared in concentrations of 10 mg./ml. A weighed quantity of the amino-acid was dissolved in a minimum quantity of 20% (w/v) HCl, and the solution was made to volume with distilled water. From these stock solutions, test solutions were made as desired.

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Table 1. *Effect of high-voltage cathode rays on aqueous solutions of amino-acids*

Amino-acid and dose of cathode rays (r.e.p.)	Percentage retention of amino-acid in solutions. Amino-acid concn. (μg./ml.) 100	250	500	1000
L-Cystine				
Control	100·0	100·0	100·0	100·0
100000	45·8	72·2	92·0	97·7
250000	38·2	66·6	79·9	95·0
500000	22·7	53·7	70·0	90·2
1000000	0·0	35·7	61·3	82·0
DL-Phenylalanine				
Control	100·0	100·0	100·0	100·0
100000	56·5	76·8	88·1	91·6
250000	23·6	57·4	75·6	86·4
500000	3·1	33·1	57·9	79·6
1000000	0·0	9·6	35·3	70·8
L-Tyrosine				
Control	100·0	100·0	100·0	100·0
100000	50·1	77·6	79·3	88·4
200000	42·3	69·3	74·1	88·3
400000	27·9	55·5	71·4	83·9
800000	6·9	34·1	59·4	76·6
	Amino-acid concn. (μg./ml.)			
DL-Tryptophan	50	100	250	500
Control	100·0	100·0	100·0	100·0
100000	46·4	81·0	92·2	94·8
250000	43·0	68·6	91·2	97·0
500000	30·0	58·8	77·6	90·0
1000000	10·0	36·6	62·2	79·4

Table 2. *Ionic yields of the decomposition, by high-voltage cathode rays, of amino-acids in aqueous solutions*

Amino-acid and dose (r.e.p.)	Ionic yields* in solutions. Amino-acid concn. (μg./ml.) 100	250	500	1000
L-Cystine				
100000	0·85	1·08	0·62	0·36
250000	0·39	0·52	0·62	0·31
500000	0·24	0·36	0·47	0·31
1000000	—	0·25	0·30	0·28
DL-Phenylalanine				
100000	0·99	1·32	1·35	1·90
250000	0·69	0·97	1·10	1·24
500000	0·44	0·76	0·96	0·93
1000000	—	0·51	0·74	0·66
L-Tyrosine				
100000	1·03	1·16	2·77	2·40
200000	0·60	0·80	1·35	1·20
400000	0·38	0·56	0·74	0·84
800000	0·24	0·43	0·53	0·60
	Amino-acid concn. (μg./ml.)			
DL-Tryptophan	50	100	250	500
100000	0·49	0·35	0·36	0·48
250000	0·21	0·23	0·14	0·11
500000	0·13	0·15	0·21	0·19
1000000	0·08	0·12	0·17	0·19

* Ionic yield = molecules of amino-acid destroyed per ion pair; 1 r.e.p. = $1{\cdot}6 \times 10^{12}$ ion pairs/g.

A pressure-insulated Van de Graaff electrostatic generator (Trump & Van de Graaff, 1948) operating at 3000000 V. was used for the cathode-ray irradiation, the technique for irradiation being the same as that previously described (Bhatia & Proctor, 1951). Each amino-acid was irradiated in four concentrations at four dose levels.

Microbiological determinations of the amino-acids were made with *Leuconostoc mesenteroides* P-60 as the test organism. For the estimation of cystine, tryptophan, and phenylalanine, the basal medium of Dunn, Shankman, Camien, Frankl & Rockland (1944) was used, and for tyrosine, the basal medium developed by Steele, Sauberlich, Reynolds & Baumann (1949).

RESULTS

No colour change was observed in the irradiated solutions of cystine. A dull-yellow pigmentation was produced in the irradiated solutions of tryptophan, phenylalanine and tyrosine. The intensity of the colour increased progressively with dose at a given concentration and also with concentration at a given dose of irradiation. When the irradiated solutions were allowed to stand overnight in the refrigerator, the intensity of the colour did not change except with tryptophan, where the pigmentation became more intense on standing.

The characteristic odour of hydrogen sulphide was noticed in all the irradiated solutions of cystine. The presence of hydrogen sulphide was confirmed by the lead acetate test. The intensity of the dark spot on the lead acetate paper, inserted into the bottle of irradiated solution, increased progressively with cathode-ray dose at a given concentration. No attempt was made to determine the evolution of hydrogen sulphide quantitatively.

Table 3. *Decomposition of* DL-*phenylalanine by high-voltage cathode rays*

(G value = number of molecules reacting/100 eV. (electron volts) of energy.)

Concentration of solution, C (g./ml.)	Inactivation dose, D (r.e.p.)	Specific-inactivation dose D/C (r.e.p./g./ml.)	Average G value
$1{\cdot}0 \times 10^{-4}$	$1{\cdot}64 \times 10^{5}$	$1{\cdot}64 \times 10^{9}$	4·26
$2{\cdot}5 \times 10^{-4}$	$4{\cdot}27 \times 10^{5}$	$1{\cdot}70 \times 10^{9}$	4·09
$5{\cdot}0 \times 10^{-4}$	$8{\cdot}89 \times 10^{5}$	$1{\cdot}78 \times 10^{9}$	3·93

The percentage retention of the amino-acids in the aqueous solutions after irradiation is shown in Table 1. The cathode-ray doses are expressed in terms of roentgen-equivalent-physical (r.e.p.), as described by Evans (1947) 1 r.e.p. = 83 ergs/g.

The ionic yields of the decomposition of amino-acids by cathode rays (calculated from the data in Table 1) are shown in Table 2.

From the data in Table 1, calculations have been made of the inactivation dose, the specific-inactivation dose, and the G values (G value = number of molecules reacting/100 eV. of energy) (Table 3) for phenylalanine at three concentrations. The pH values of all solutions before irradiation are given in Table 4.

Table 4. pH *of solutions of amino-acids prior to irradiation*

Amino-acid	Amino-acid concn. (μg./ml.) 100	250	500	1000
	pH of amino-acids in solutions			
L-Cystine	2·90	2·59	2·39	2·14
DL-Phenylalanine	2·84	2·50	2·25	2·00
L-Tyrosine	2·61	2·31	2·05	1·80
DL-Tryptophan*	2·78	2·44	2·21	—

* At a concentration of 50 μg./ml. the pH was 2·94.

DISCUSSION

All the amino-acids were decomposed upon irradiation, and the decomposition was related exponentially to the dose. The difference in the amino-acid content of the solution before and after irradiation is considered as the amount decomposed. The dilute solutions were relatively more affected by a given dose than were the more concentrated solutions.

The ionic yields of the decomposition of L-cystine over a range of concentrations of from 100 to 1000 μg./ml. were (with a single exception) less than 1·00. The ionic yields of the decomposition of DL-tryptophan over a range of concentrations of from 50 to 500 μg./ml. were less than 1·00 in all instances. For tyrosine, ionic yields greater than 1·00 were obtained at all concentrations when the irradiation dose was 100000 r.e.p., and at concentrations of 500 and 1000 μg./ml. when the dose was 200000 r.e.p. The ionic yields in the more dilute solutions were less than 1·00. The ionic yields of the decomposition of phenylalanine were less than 1·00 except in a few solutions containing 250, 500, and 1000 μg. of the amino-acid/ml.

It will be noted that the ionic yields, as measured microbiologically by inactivation of cystine, is of the order 0·26–0·36 for a solution with a concentration of 1000 μg./ml. This agrees with the value of 0·2 found by Dale & Davies (1951) for a cysteine solution of the same concentration (based on hydrogen sulphide production). The reason that Dale & Davies found no liberation of hydrogen sulphide from cystine may be because the irradiation doses they used were much lower than those used in the present investigation.

The inactivation dose for phenylalanine was found to be dependent on its concentration in solution over a range of concentrations of from 100 to 500 μg./ml., but the specific inactivation doses were constant. Under the experimental conditions studied, the G values were of the order of four

molecules reacting/100 eV., which agrees with the values given by Allen (1947) for organic liquids.

These observations are all indicative of an indirect action of high-voltage cathode rays on these amino-acids. For ionic yields higher than 1·00, the same explanation as given previously for histidine (Bhatia & Proctor, 1951) would probably be valid, namely, that molecular fragments of the amino-acids resulting from cathode-ray treatment competed for the free radicals.

Evolution of hydrogen sulphide from cystine indicates that the molecule of cystine is probably decomposed at the disulphide linkage.

SUMMARY

1. Aqueous solutions of L-cystine, DL-phenylalanine, L-tyrosine, and DL-tryptophan in various concentrations were irradiated with high-voltage cathode rays at doses ranging from 10^5 to 10^6 r.e.p. Determinations of the amino-acid contents of the solutions were made microbiologically after irradiation.

2. All four amino-acids were decomposed upon irradiation, and the decomposition was related exponentially to the dose.

3. Ionic yields were determined for the four amino-acids over a wide range of concentrations.

4. Data are presented to show that the action of high-voltage cathode rays on these amino-acids took place through free radicals being intermediates.

5. Evolution of hydrogen sulphide from the cystine solution indicated that cystine was probably decomposed at the disulphide linkage upon irradiation with high-voltage cathode rays.

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Some Chemical Properties of Helvolic Acid

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Burton & Abraham (1952) reported that cephalosporin P_1, an antibiotic produced by a species of *Cephalosporium*, resembled helvolic acid (Chain, Florey, Jennings & Williams, 1943). Further work by Burton, Cardwell & Abraham (1951) has confirmed this resemblance and has allowed a simple interpretation of the results of a chemical investigation into helvolic acid, completed by the author in 1945 and described in this paper.

The isolation of helvolic acid from culture filtrates of *Aspergillus fumigatus* mut. *helvola* Yuill has been described by Chain *et al.* (1943). Helvolic acid was shown to be a colourless crystalline compound, melting point 212°. It is readily soluble in most organic solvents, except light petroleum, but is insoluble in water; its sodium salt is readily soluble in water. Helvolic acid was assigned the formula $C_{32}H_{44}O_8$. It titrated as a monobasic acid and a monomethyl ester was prepared by the action of diazomethane.

These preliminary observations on the chemical properties of helvolic acid were confirmed and extended in the investigation now described, which was directed primarily to the discovery of the role of the oxygen atoms in the molecule.

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The helvolic acid required in the present investigation was produced by the method previously described (Chain *et al.* 1943). From a total of 5200 l. of crude culture filtrate, worked up in twenty-seven batches, 5·5 g. of pure helvolic acid were obtained. Attempts to increase this yield by varying the culture medium and the conditions of growth were unsuccessful.

An investigation of the rate of development of anti-bacterial activity, assayed by the ordinary plate-and-cylinder method used for penicillin, showed that when the mould was grown as a surface culture at 25°, in a synthetic medium of glucose and mineral salts, a peak activity was reached after about 8 days' growth. Thereafter, the activity fell considerably to a minimum at 16–18 days. This was followed by a second increase to a fairly constant value at 21 days, at which time the crop was harvested for the isolation of helvolic acid: it was found that the antibacterial activity of cultures of 6–8 days' growth was due, not to helvolic acid, but to gliotoxin. An account of this work has been published elsewhere (Glister & Williams, 1944).

Waksman, Horning & Spencer (1943) described an antibiotic, which they named fumigacin, produced by *A. fumigatus* Fres. Menzel, Wintersteiner & Hoogerheide (1944) showed that fumigacin was a mixture of gliotoxin and helvolic acid; their account of helvolic acid, as far as it goes, agrees in almost all essentials with the findings of the present author.

A point of discrepancy, however, is the value of the specific rotation. Chain *et al.* (1943) gave the value $[\alpha]_D^{20°} - 49{\cdot}4°$ in chloroform; Menzel *et al.* gave $[\alpha]_D^{28°} - 132° \pm 2°$. Two separate, highly purified, samples have now been found to give $[\alpha]_D^{23°} - 113°$ (*c*, 3·1) and $-117°$ (*c*, 2·6) respectively. The difference still existing may be due to contamination with gliotoxin of the material used by these workers; gliotoxin is strongly laevorotatory ($[\alpha]_D^{25°} - 254°$). The possibility of contamination was raised by Menzel *et al.* and not entirely disproved. Their product reduced Fehling's solution slowly at 100°. Our purified samples have no action on Fehling's solution even on heating for 1 hr. at this temperature. Gliotoxin, however, readily reduces Fehling's solution.

RESULTS

Nature of oxygen atoms. All the analytical and degradative evidence supports the formula $C_{32}H_{42-44}O_8$. For helvolic acid, Menzel *et al.* proposed a formula $C_{29}H_{38-40}O_7$; several objections to this will be discussed later.

The absence of enolic or phenolic properties in helvolic acid show that the acidic properties are due to a carboxyl group. This is confirmed by the preparation of a monomethyl ester (m.p. 262°) by the action of diazomethane on the acid. Analysis of the ester gave results in conformity with a formula $C_{33}H_{44-46}O_8$. The molecular weight was found by an X-ray crystallographic method (Crowfoot & Low, 1943) to be 556 ± 14 ($C_{33}H_{46}O_8$ requires 570).

A monosemicarbazone and a dioxime of helvolic acid, and a monosemicarbazone of its methyl ester, have been prepared. The absence of reducing properties and the positive Zimmermann reaction indicate that at least one of the carbonyl groups is ketonic. Menzel *et al.*, using a different preparative method, obtained a monoxime.

An acetoxyl determination by the standard (acid hydrolysis) method of Elek & Harte (1936) gave values of 8·3 and 7·8% acetoxyl for the free acid and 7·9% for the methyl ester. Menzel *et al.* record similar results for this method. The calculated values, assuming the formula $C_{32}H_{44}O_8$ for the acid, are 7·74 and 7·54% respectively for one acetoxyl group. The volatile acid was identified as acetic acid. Drs Weiler and Strauss (Dyson Perrins Laboratory, Oxford) carried out an acetoxyl determination on helvolic acid by hydrolysing it for 15 min. at 100° with 0·1N-ethanolic sodium hydroxide. After acidification, the volatile acids were removed by steam distillation and collected in excess of standard alkali. They obtained values of 16·3 and 15·6% acetoxyl, i.e. roughly twice those given by the method of Elek & Harte. These results suggest that, while helvolic acid contains two acetoxyl groups, one is much more readily split off than the other. Since helvolic acid is virtually insoluble in acid solution, the conditions of the Elek & Harte method of estimation do not favour the removal of an unreactive acetoxyl group.

If helvolic acid is hydrolysed in 0·1N-alkali for 36–48 hr. at 37°, approximately one equivalent of alkali is taken up, in addition to that required to neutralize the carboxyl group, showing the liberation of a second acidic grouping. This probably corresponds to hydrolysis of the more reactive of the two acetoxyl groups. A crystalline sodium salt of the new acid is precipitated during hydrolysis. The corresponding acid has been obtained in a crystalline form. Its equivalent weight by direct titration was 590; on again treating with excess alkali at 37°, its equivalent was very nearly half this, namely 298. Further hydrolysis with alkali gave, after acidification, a substance which no longer dissolved readily in alkali and which analysed for $C_{28}H_{36}O_5$. This suggests that hydrolysis of the second acetoxyl group gives an hydroxyl so situated that it readily lactonizes with the free carboxyl group (Burton *et al.* 1951).

On the evidence adduced above, all the eight oxygen atoms known to be present in the helvolic acid molecule may be satisfactorily accounted for. Two occur in a carboxyl group, two in carbonyl groups, and the remaining four are distributed

between two acetoxyl groups, one of which is much more resistant than the other to acid hydrolysis. The hydroxyl group liberated on hydrolysis of this second acetoxyl group is so situated that it readily lactonizes with the carboxyl group.

Reaction with bromine. Helvolic acid behaves as an unsaturated substance, decolorizing bromine water and permanganate in the cold and reacting readily with ozone. When the acid is treated in the cold with excess bromine in glacial acetic acid, four atoms of bromine react per molecule of acid, but the low bromine content of the product indicates that the main reaction here is oxidative.

Oxidation with hydrogen peroxide. Helvolic acid is readily and smoothly oxidized by hydrogen peroxide (perhydrol) or by periodic acid, to the same neutral compound, m.p. 229–230°, the properties of which are consistent with a formula $C_{32}H_{46}O_{11}$.

One possible explanation is that this compound results from the formation of a diglycol at the site of two double bonds, followed by loss of water to form a lactone ring.

$$C_{32}H_{42-44}O_8 \longrightarrow C_{32}H_{46-48}O_{12} \xrightarrow{-H_2O} C_{32}H_{44-46}O_{11}.$$

On prolonged hydrolysis at 37° with 0·1 N-alkali, the compound slowly dissolves; its equivalent weight under these conditions is 160. The molecular weight corresponding to $C_{32}H_{46}O_{11}$ is 606 and thus four equivalents of alkali are apparently involved in the alkaline hydrolysis of the perhydrol oxidation product; this has been characterized as a dioxime (additional evidence for the presence of two carbonyl groups in helvolic acid itself) and as the benzoyl and 3:5-dinitrobenzoyl esters. With ketene, a monoacetyl derivative was obtained. In view of the evidence that helvolic acid itself contains two acetoxyl groups, it seems that the two remaining hydroxyl groups believed to be present in the perhydrol product are, therefore, rather inert.

Further oxidation with lead tetraacetate. Recognition of the presence of two double bonds led to attempts to split up the molecule by attacking it at these points. With cold alkaline permanganate an immediate reaction takes place, involving ten atoms of oxygen per molecule; there is a further slow reaction in which more than twenty oxygen atoms per molecule are involved. Oxidation with chromium trioxide appears to follow a similar course. It was not possible, however, to characterize any degradation products. With lead tetraacetate, a neutral crystalline derivative was obtained; melting point above 200° (decomp.). Analysis indicated a formula $C_{32}H_{44}O_{11}$; the molecular weight by X-ray crystallography (Crowfoot & Rogers, 1944) is 595 ± 15 ($C_{32}H_{44}O_{11}$ requires 604).

Helvolic acid and its methyl ester react readily with ozone. The acid itself appears to yield two acidic fractions; the methyl ester yields a neutral product. The products could not, however, be characterized.

Helvolic acid is rapidly oxidized by selenium dioxide in ethanol at 100°, but again it was not possible to isolate any degradation product.

The possibility of dehydrogenation with selenium was explored, but preliminary experiments, and the experience of other workers with different substances, indicate that this method is not likely to be fruitful unless the oxygen content of the molecule can first be reduced.

Reduction. Helvolic acid is very resistant to reduction; after attempted reduction with phosphorus and iodine (6 hr. at 100°) unchanged acid was recovered in good yield.

DISCUSSION

So far as common ground is covered, the results described here are, in almost all respects, in agreement with those of Menzel *et al.* (1944). On questions of fact they differ significantly only in the finding that helvolic acid has no reducing properties. The evidence is entirely consistent with a formula $C_{32}H_{42-44}O_8$ for helvolic acid. Menzel *et al.* are inclined to favour a formula $C_{29}H_{38-40}O_7$, but there are several objections to this. In particular, it corresponds to a molecular and equivalent weight (498 or 500) well outside the limits (562 ± 12) set by the X-ray crystallographers (Crowfoot & Low, 1943). The American workers themselves record equivalent weights of 514, 560 and 591; their molecular weights were determined by the Rast method, which is very much less reliable than X-ray crystallography. A further objection to the C_{29} formula is that we have found good evidence for the existence of eight oxygen atoms in the molecule. The analytical results obtained by Menzel *et al.* agree almost equally well with either formula, but for the silver salt they record values practically identical with the theoretical values for $C_{32}H_{43}O_8Ag$.

The eight oxygen atoms required by the formula $C_{32}H_{44}O_8$ can be satisfactorily accounted for as follows: two in a carboxyl group; four in acetoxyl groups; two in carbonyl groups (at least one ketonic). Helvolic acid behaves as an unsaturated compound, and the course of the reaction with perhydrol, as well as other evidence, suggests that two double bonds are present. The fact that the perhydrol oxidation product is a neutral compound, and that there is evidence that it contains a lactone ring, makes it probable that one double bond is in a position γ or δ to the carboxyl group.

No direct evidence has been obtained about the kind of carbon skeleton present in the molecule. Dr R. E. Richards, of the Physical Chemical Laboratory, Oxford, investigated the infrared

spectrum of helvolic acid and certain of its derivatives. The complexity of the spectrum, which can be attributed to the number of unsaturated carbon-oxygen linkages, prevents many definite conclusions being reached, but the spectra can be satisfactorily interpreted on the lines of the conclusions reached from chemical evidence. Of particular interest is the absence of absorption corresponding to aromatic nuclei. It appears, therefore, that helvolic acid has a partially unsaturated, alicyclic structure.

EXPERIMENTAL

Unless otherwise stated, elementary analyses and determinations of molecular weight by the Rast method were made by Drs Weiler and Strauss, Dyson Perrins Laboratory, Oxford.

Optical activity. Determinations were made with two highly purified samples of helvolic acid with the following results: specimen 1 (*c*, 3·1 in $CHCl_3$), $[\alpha]_D^{23°} = -113°$; specimen 2 (*c*, 2·6 in $CHCl_3$), $[\alpha]_D^{23°} = -117°$. For methyl helvolate (*c*, 2·6 in $CHCl_3$) a value $[\alpha]_D^{22°} = -133°$ was found.

Carbon-methyl determination. Found 11·1% *C*-Me. $C_{32}H_{44}O_8$ requires 9·7% for two *C*-Me groups and 14·5% for three.

Carboxyl derivatives

Helvolic acid semicarbazone. This was prepared by a method described by Hopper (1929). Helvolic acid (21·6 mg.) was dissolved in 0·5 ml. of pure pyridine and 11·5 mg. of semicarbazide hydrochloride were added as a concentrated aqueous solution. The mixture was kept at room temperature for 16 hr. On diluting the mixture with water, the almost pure semicarbazone slowly crystallized out. It was recrystallized from ethanol (75%, w/v). Yield 12·1 mg. *Helvolic acid semicarbazone* had m.p. 240–250° (decomp.). (Found: C, 64·6; H, 7·3; N, 6·9. $C_{32}H_{47}O_8N_3$ requires C, 64·6; H, 7·7; N, 6·9%.)

Helvolic acid dioxime. This was prepared by a method similar to that used for the semicarbazone except that the reaction was carried out in a sealed tube at 100°, heating being continued for 3 hr. The product crystallized from ethanol in long needles. *Helvolic acid dioxime* had m.p. 189°. (Found: C, 65·1; H, 7·6; N, 4·0, 4·7. $C_{32}H_{46}O_8N_2$ requires C, 65·6; H, 7·8; N, 4·8%.)

Methyl helvolate semicarbazone. This was prepared in exactly the same way as the semicarbazone of helvolic acid. It crystallizes from ethanol in long needles. *Methyl helvolate semicarbazone* had m.p. 212°. (Found: C, 65·2; H, 7·7; N, 5·6. $C_{34}H_{49}N_3O_8$ requires C, 65·1; H, 7·8; N, 6·7%. Lack of material prevented repetition of the determination of N.)

Silver helvolate

This was prepared by the method described by Menzel *et al.* (1944). Helvolic acid (56·7 mg.) was dissolved in 5 ml. ethanol, and the solution was neutralized by addition of the calculated quantity of 0·1 N-NaOH. 10 ml. of 1% aqueous $AgNO_3$ were added. The precipitated Ag salt was washed with water, dried, and recrystallized by precipitation from $CHCl_3$ solution by addition of ether. The freshly prepared salt is colourless; it darkens on keeping. (Found: C, 58·2; H, 6·5; Ag, 15·3. $C_{32}H_{43}O_8Ag$ requires C, 57·9; H, 6·5; Ag, 16·3%.)

Acetyl determinations

Method of Elek & Harte (1936)

The experimental procedure prescribed by these authors was rigidly followed.

Helvolic acid (two samples, hydrolysed for 65 and 70 min. respectively) contained 8·26 and 7·76% acetyl; $C_{32}H_{44}O_8$ requires 7·74% acetyl for 1 Ac/molecule. Methyl helvolate (hydrolysed for 80 min.) contained 7·94% acetyl; $C_{33}H_{46}O_8$ requires 7·54% acetyl for 1 Ac/molecule.

Identification of volatile acid formed in Elek & Harte determination. A method designed by Feigl (1937) was used. The volatile acid from 10·5 mg. of helvolic acid was collected in distilled water at 0° and the solution neutralized with excess $CaCO_3$. After evaporating to dryness, the residue was heated in a small tube and the vapour evolved allowed to fall on paper moistened with a saturated solution of *o*-nitrobenzaldehyde in 2 N-NaOH. The blue colour of indigo developed. The method failed to detect 0·3 mg. of acetic acid, but gave a decisive colour with 0·9 mg.

Determination by alkaline hydrolysis

This determination was made independently by Drs Weiler and Strauss, Dyson Perrins Laboratory, Oxford. Helvolic acid was hydrolysed for 15 min. at 100° with 0·1 N-ethanolic NaOH. The reaction mixture was acidified with H_2SO_4, steam distilled, and the volatile acids were absorbed in excess standard alkali. Values of 16·3 and 15·6% acetoxyl were found for two separate samples.

Alkaline hydrolysis at 37°

The acid (two samples hydrolysed for 56 and 144 hr. respectively) absorbed 2·28 and 3·52 equivalents of alkali.

Helvolic acid was dissolved in 3 ml. of 0·1 N-NaOH and the residual alkali was titrated after a known interval.

When the first titration above had been completed, excess acid was added, when a white curdy precipitate appeared. This was taken up in $CHCl_3$, and the $CHCl_3$ layer repeatedly washed with distilled water until the washings were neutral. The $CHCl_3$ solution was then evaporated to dryness and the equivalent of the amorphous acidic residue was determined by direct titration with the micro-burette (Linderstrøm-Lang & Holter, 1931). (Found. C, 70·5; H, 8·2; equiv. 566, 615 (mean 590). $C_{30}H_{46}O_7$ requires C, 70·3; H, 7·9%; equiv. 513.)

The hydrolysis product (29·3 mg.) was dissolved in 3 ml. of 0·1 N-NaOH and kept at 37° for 48 hr. The equivalent weight was then determined by estimating the remaining alkali with 0·1 N-HCl; equiv. found 298. Hydrolysis of helvolic acid with 0·1 N-NaOH at 100° gave on acidification a material which was not readily soluble in alkali and was presumed to be a lactone. (Found C, 74·5; H, 8·8. $C_{28}H_{36}O_5$ requires C, 74·3; H, 8·0%.)

Reaction with bromine

When treated with excess 0·01 N-Br_2 in glacial acetic acid at room temperature, helvolic acid reacted with 3·71 and 3·81 atoms of Br/molecule respectively in two separate experiments. A non-crystalline product containing about 5% Br was isolated.

Reaction of helvolic acid with perhydrol and with periodic acid

Oxidation proceeds smoothly, giving the same product in either case. The following procedures are typical:

(1) Helvolic acid (65·9 mg.) was dissolved in 7 ml. of glacial acetic acid and 1 ml. perhydrol added. The mixture was allowed to stand for 16 hr. at room temperature and then evaporated to dryness in an evacuated desiccator. The residue was crystallized from 70% acetic acid. Yield 28·8 mg.

(2) The reagent was a 2·5% solution of sodium periodate in 75% acetic acid. Helvolic acid (23·6 mg.) was dissolved in 2·5 ml. of reagent and solution allowed to stand for 40 hr. at 37°. The mixture was diluted with 5 ml. of water and the precipitate dissolved in $CHCl_3$. The $CHCl_3$ layer was separated, washed and evaporated at room temperature. The residue was recrystallized from 70% acetic acid. Yield 10 mg.

The identity of the product obtained by the two methods was proved by analysis, identity of melting points, and mixed, melting point. It was finally proved (Crowfoot & Rogers, 1944) by the complete identity of the X-ray crystallographic pictures. The product is neutral, crystallizing from acetic acid as small, colourless needles, m.p. 229–230°. It is insoluble in water. (Found: C, 64·4, 63·0, 64·4; H, 7·6, 7·6, 8·0; mol.wt. (Rast), 560. $C_{32}H_{46}O_{11}$ requires C, 63·4; H, 7·6; mol.wt. 606.) The perhydrol product dissolves slowly in 0·1 N-NaOH. 15·5 mg., treated for 7 days at 37° with 0·1 N-NaOH, went completely into solution. The quantity of alkali used up corresponded to an equivalent of 160.

A dioxime was prepared by the method used for helvolic acid. (Found: N, 4·3. $C_{32}H_{48}O_{11}N_2$ requires N, 4·4%.)

By reaction with excess ketene in ethereal solution at room temperature, a crystalline monoacetyl derivative was obtained; m.p. above 225° (decomp.). (Found: for two specimens C, 64·8, 64·2; H, 7·6, 7·6; Ac, 22·3. $C_{34}H_{48}O_{12}$ requires C, 64·0; H, 7·4; Ac, 19·8%.) By reaction with benzoyl chloride for 16 hr. in pyridine solution at room temperature, a crystalline monobenzoyl derivative was obtained, m.p. 248°. (Found: C, 66·8; H, 6·4. $C_{39}H_{50}O_{12}$ requires C, 65·9; H, 7·0%.)

A 3:5-dinitrobenzoyl derivative was prepared in the same way, m.p. above 215° (decomp.). (Found: N, 3·6. $C_{39}H_{48}O_{16}N_2$ requires N, 3·5%.)

Further oxidation of perhydrol oxidation product with lead tetraacetate. The reagent used was a saturated solution of lead tetraacetate in glacial acetic acid. Helvolic acid (92·9 mg.) was converted to perhydrol oxidation product, and the latter was dissolved in 5 ml. of the tetraacetate reagent. After standing at 37° for 16 hr. the mixture was diluted with 5 vol. distilled water and shaken for 3 hr. to decompose excess tetraacetate. The solution was extracted with 5 ml. $CHCl_3$ and the $CHCl_3$ extract evaporated to dryness. The residue (72·8 mg.) was crystallized twice from hot ethanol. A neutral product was obtained, crystallizing in short needles, melting point indeterminate (decomp.). (Found: for two specimens C, 62·1, 63·5; H, 6·8, 7·2. $C_{32}H_{44}O_{11}$ requires C, 63·6; H, 7·3%.) Molecular weight was found by X-ray crystallography (Crowfoot & Rogers, 1944) to be 595 ± 15 ($C_{32}H_{44}O_{11}$ requires mol.wt., 604).

SUMMARY

1. All analytical and degradative evidence suggests that helvolic acid has the formula $C_{32}H_{42-44}O_8$. It titrates as a monobasic carboxylic acid.

2. The following derivatives of helvolic acid have been prepared and characterized: the semicarbazone, dioxime, methyl ester, silver salt, and a product obtained by oxidation with either perhydrol or periodic acid. The latter appears to be a lactone.

3. From the methyl ester, a semicarbazone has been prepared. From the perhydrol oxidation product, a dioxime, an acetoxyl, a benzoyl, and a 3:5-dinitrobenzoyl derivative have been prepared.

4. The behaviour of helvolic acid and certain of its derivatives on alkaline hydrolysis has been studied. Results indicate the presence of two acetoxyl groups in the original molecule.

5. Helvolic acid behaves as an unsaturated substance containing two double bonds.

It is a pleasure to record my thanks to Dr E. Chain, F.R.S., now of the Istituto Superiore di Sanità, Rome, for his encouragement and advice while the original experimental work was being done.

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The Effect of Thyroxine and Thiouracil on the Composition of Milk

3. THE CONCENTRATION OF THE MAJOR CONSTITUENTS AND OF SOME OF THE WATER-SOLUBLE VITAMINS IN COW'S MILK

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When lactating animals are treated with thyroxine, milk yield is almost invariably markedly stimulated (Graham, 1934*a*, *b*; Jack & Bechdel, 1935; Folley & White, 1936; Herman, Graham & Turner, 1938; Smith & Dastur, 1940; Owen, 1948*a*; Chanda & Owen, 1951). Usually the yield of fat is stimulated to a greater extent than that of the milk so that an increase in fat percentage results. This latter effect is, however, not always observed. In some experiments the solids-not-fat of the milk increase (Folley & White, 1936), but this again is not a constant finding (Archibald, 1945). The changes in the total nitrogen content of the milk caused by iodinated casein or thyroxine are also highly variable. Thus Ralston, Cowsert, Ragsdale, Herman & Turner (1940) reported a decline in the total nitrogen content of the milk. On the other hand, Van Landingham, Hyatt & Weakley (1946), Hibbs & Krauss (1947), Owen (1948*a*) and Chanda & Owen (1951) found no change in the total nitrogen content or in the partition of nitrogen in the milk. It came to be generally believed that, apart from its effect on fat, thyroxine caused only minor changes in the composition of the milk. Owen (1948*a*), however, found a significant increase in the phosphorus content of the milk without any concomitant change in either the nitrogen or the calcium contents. Chanda & Owen (1951) found that the goitrogenic substance thiouracil caused a decrease in the total phosphorus of milk without any change in its nitrogen content, and in the same experiments they showed that thyroxine increased the ester phosphorus and lipid phosphorus while decreasing the inorganic phosphorus in cow's milk. They also found that thiouracil produced changes in the partition of phosphorus which were the reverse of those produced by thyroxine. Changes in phosphorus partition, whether produced by thyroxine or thiouracil, were inversely correlated with changes in milk phosphatase. In the present paper the effects of thyroxine and thiouracil on the partition of aneurin in relation to changes in the phosphatase titre of the milk are described. The simultaneous changes produced in the concentrations of some of the major constituents of milk and in the concentrations of certain minerals and water-soluble vitamins were also followed.

EXPERIMENTAL

There were five experiments, each divided into periods 1–3, the hormonal treatments being confined to period 2. Each period lasted 3 weeks, except for period 2 of Exp. 5 which was a fortnight. Three cows were used in Exp. 1, and six in each of the other four experiments. In Exp. 1, there was one control cow, one treated subcutaneously with 10 mg. DL-thyroxine per day, and a third which received, subcutaneously, 10 mg. thiouracil per day. In each of the other four experiments two cows received 10 mg. DL-thyroxine per day each, two received 20 mg. thiouracil per day each, and two were controls. For the purpose of calculating the responses to the drugs, milk yields of all the cows were recorded. In Exp. 2, however, the milk of one of the control cows was not chemically analysed. In Exp. 3, aneurin in the milk was partitioned and the milk was also analysed for riboflavin, creatine, creatinine, Ca, Mg, Na and K. Haemoglobin, and red and white cell counts were determined in the blood of cows in Exp. 3. Fat, solids-not-fat, ascorbic acid and phosphatase were determined in the milk in Exps. 1–3. In addition, in Exps. 1 and 2, lactose, chloride and freezing point were determined. The milk yields only are considered in Exps. 4 and 5. In Exp. 1, 10 mg. thiouracil were found to cause the milk phosphatase to increase at only half the rate at which 10 mg. thyroxine caused it to decrease (Chanda & Owen, 1951). The dose of thiouracil was therefore increased to 20 mg. in all subsequent experiments. 20 mg. thiouracil gave an increase of the same magnitude as the decrease caused by 10 mg. thyroxine (Chanda & Owen, 1951). Thyroxine was dissolved as described by Folley & White (1936) prior to injection. The solution contained 1 mg. DL-thyroxine sodium B.P. (British Drug Houses Ltd.) per ml. Since thiouracil, like thyroxine, is soluble in alkali, it was possible to prepare a solution of it in the same way. A weighed amount of thiouracil was dissolved in a minimal excess of 0·1 N-NaOH and neutralized to phenolphthalein with 0·1 N-HCl. The solution was then diluted with distilled water to 1 mg./ml. in Exp. 1, or 2 mg./ml. in the other experiments. The solutions of the drugs were preserved in the refrigerator. Samples for filling 10 ml. syringes for injection were 'flash pasteurized' in weighing bottles immediately before use by having their temperature momentarily raised to 80°.

Methods of analysis

Fat was determined by the Gerber method (British Standards Institution, 1936). *Total solids* were determined by drying a weighed sample of the milk first on a water bath and then in an electric oven at 100°. *Solids-not-fat* were determined from the difference between total solids and fat, but were calculated to a fat-free basis. *Protein* was calcu-

lated by multiplying the total nitrogen by the factor 6·38. *Lactose* was determined polarimetrically by Vieth's method as described by Elsdon & Walker (1942), the necessary correction being made for the volume of the precipitate. *Chloride* was estimated by the method of Davies (1938). The *freezing point* was estimated by the improved Hortvet apparatus described by Temple (1937). *Calcium*, *magnesium* and *sodium* were estimated in milk samples which were evaporated in vitreosil basins on a water bath prior to being ashed in an electric muffle furnace which was thermostatically controlled at 600°. The ash was dissolved in warm 6 N-HCl and made up to a known volume. The final stage in the estimation of calcium was the titration of the precipitated calcium oxalate with $KMnO_4$ as described by Hawk, Oser & Summerson (1947). Sodium was precipitated as sodium zinc uranyl acetate in sintered-glass filters, dried at 100° and weighed (Peters & Van Slyke, 1932). Magnesium was determined from the phosphate content (Fiske & Subbarow, 1925) of a magnesium ammonium phosphate precipitate. *Potassium* was precipitated as cobaltinitrite from a 2% trichloroacetic acid (TCA) filtrate of whole milk and the cobalt in the centrifuged and washed precipitate was determined by its colour reaction with nitroso-R-salt (Sideris, 1942). The potassium figures were corrected for the volume of the protein precipitated by TCA (Rowland, 1938) and for co-precipitated sodium. *Creatinine* was determined by treatment of the tungstic acid filtrate of milk with alkaline picrate solution, the resulting colour being read in a Spekker absorptiometer. *Creatine* was determined by subtracting the preformed creatinine from the total creatinine determined by the same method after autoclaving the protein-free filtrate with HCl (Hawk *et al.* 1947). *Phosphatase* was determined as already described by Chanda & Owen (1951). *Ascorbic* acid was estimated according to the method used by Mattick *et al.* (1945), precaution being taken that the samples of milk were made large enough to fill the preserving jars in which they were taken so as to prevent any aerobic oxidation. The milk was pipetted into the TCA-metaphosphoric acid mixture within 15 min. of the collection of the samples, to avoid formation of any dehydroascorbic acid. *Riboflavin* was determined in a Spekker fluorimeter by the method of Emmerie (1938) combined with Arnold's (1945) modification. *Aneurin* was determined fluorimetrically in *iso*butanol as thiochrome. The aneurin was partitioned as described by Houston, Kon & Thompson (1940). Free aneurin was determined in skimmed milk by Jansen's (1936) method, while cocarboxylase was determined from the difference between free aneurin and aneurin present in the takadiastase digest of the TCA extract of skim milk. Pepsin digestion of the TCA precipitate gave the protein-bound aneurin. Total aneurin (free plus cocarboxylase plus protein-bound) was independently determined by digestion of skim milk with takadiastase only, for it was found in confirmation of Houston *et al.* (1940) that pepsin digestion following takadiastase digestion did not materially increase the total.

RESULTS AND DISCUSSION

Milk yield. Typical graphs showing the increases in milk yield brought about by thyroxine and the decreases brought about by thiouracil have already been given (Chanda & Owen, 1951). The quantitative responses in milk yield produced by the two drugs in all the cows used in the present series of experiments are now described. In calculating the positive or negative responses in milk yield it is obviously difficult to estimate what the yield would have been had no treatment been given, since after the first few weeks from calving, the yield of milk gradually declines. The increases caused by thyroxine and decreases caused by thiouracil were therefore complicated by this natural decline. To offset this difficulty, the average daily yield (x) of each cow during period 1 was analysed by co-variance with the average daily yield (y) in period 2, the cows being divided into three treatment groups—control, thyroxine and thiouracil. From the total sum of squares and products the variance due to treatment was eliminated. From the residual sum of squares and products, the following significant regression equation was established between x and y

$$y = -0{\cdot}44 + 0{\cdot}94077x$$

(x = the actual milk yield in lb. in period 1; y = the expected milk yield in period 2). Geometrically, this method of statistical analysis corresponds to using an extrapolation of a straight line fitted to the milk yield data of period 1 as the expected yield in period 2 (Owen, Smith & Wright, 1943). Since the effects of discontinuance of the hormone treatment are just as marked as those produced by its institution, period 3 cannot be regarded simply as a second control period, so that a line fitted to the data of periods 1 and 3 would overestimate the effect of thyroxine and underestimate that of thiouracil. The expected milk yields during period 2, calculated from this equation, compared favourably with the actual milk yields shown simultaneously by each of the nine control cows. The results for all the nine thyroxine-treated cows except one showed that the actual yields in period 2 were higher than the expected yields. For the sake of brevity the mean results of each group are recorded in Table 1 which shows that the average response was +13·2% in the thyroxine-treated cows during treatment, while

Table 1. *Effect of thyroxine and thiouracil on milk yield*

Group of cows	No. of cows	Expected milk yield during period 2 calculated from the regression (lb./day)*	Actual milk yield (lb./day)	Response (%)
Control	9	21·1	21·1	—
Thyroxine (10 mg./day)	9	17·1	19·3	+13·2
Thiouracil (20 mg./day)	8	22·0	19·6	−10·7

* 1 lb. = 454 g.

the average response was −10·7 % in the eight cows treated with 20 mg. thiouracil daily. The response by the cow in Exp. 1, which received only 10 mg. thiouracil, was only −5·8 %. The responses in the individual cows within groups varied very widely and the initial yields of cows did not seem to have any effect on the relative magnitude of the response caused by either thyroxine or thiouracil.

Fat, solids-not-fat and protein content of milk. The results for solids-not-fat have been calculated to a fat-free basis. In most of the animals, thyroxine caused an increase in the fat content of the milk. The effect of the hormone on the solids-not-fat content of milk was variable. In some of the cows there was a small increase, but in others there was no change. This contrast is particularly noticeable in cow no. 12 (Exp. 3) in which the greatest response in the fat content of milk was recorded without any change in the solids-not-fat. Thiouracil decreased the fat content of milk except in cow no. 8 (Exp. 2) for which the fat content of milk was considerably increased in successive periods. The effect of thiouracil on the solids-not-fat content of the milk was inconstant.

had the opposite effect. An increase in chloride content with a corresponding decrease in lactose was also noticeable in the control cows with the progress of lactation. There was thus a close negative correlation between the average lactose and chloride contents in Table 3 ($r = -0{\cdot}9933$). Davies (1936) pointed out that since lactose and chloride account for 80 % of the osmotic pressure of milk, there tends to be a constant relationship between these two constituents. He found from analysis of numerous milk samples that the relationship can be expressed as

$$\text{lactose content} = 6{\cdot}26 - 13{\cdot}5 \times \text{chloride content},$$

or in other words lactose + 13·5 times chloride should be constant and equal to 6·26. In the present investigation this constant has been calculated and recorded in Table 3 in which it is referred to as the lactose-chloride number. It was found that this number was fairly constant for all the cows in all the periods, but its actual value was somewhat lower than that of Davies (1936). The mean value in the present experiments was found to be 6·19. In conformity with this constancy of the relationship

Table 2. *Effect of thyroxine and thiouracil on the contents of fat and solids-not-fat in milk*

Exp.	Cow	Daily injection in period 2	Fat (g./100 g. milk)			Solids-not-fat (g./100 g. fat-free milk)		
			Period 1	Period 2	Period 3	Period 1	Period 2	Period 3
1	1	None	4·07	3·75	4·15	8·78	8·89	9·15
	2	10 mg. thyroxine	3·83	4·20	5·00	8·84	9·11	9·92
	3	10 mg. thiouracil	3·82	3·23	3·38	8·46	8·21	8·57
2	4	None	4·45	4·34	4·78	8·86	8·87	9·20
	5	10 mg. thyroxine	4·51	5·08	4·83	9·45	9·96	9·61
	6	10 mg. thyroxine	4·31	4·43	4·61	8·75	8·63	8·97
	7	20 mg. thiouracil	4·06	3·62	3·46	8·48	8·53	8·52
	8	20 mg. thiouracil	4·19	4·66	5·13	8·79	8·83	9·33
3	9	None	3·95	3·90	4·24	8·92	8·89	8·78
	10	None	4·28	3·95	3·98	8·88	8·81	8·54
	11	10 mg. thyroxine	4·12	4·74	4·35	9·24	9·43	8·95
	12	10 mg. thyroxine	4·14	5·24	4·74	9·01	8·97	8·72
	13	20 mg. thiouracil	4·44	4·12	4·58	9·05	8·85	8·76
	14	20 mg. thiouracil	3·91	3·47	4·32	9·02	9·01	8·84

The protein content of the fat-free milk in the cows used in Exps. 2 and 3 confirmed the findings in Exp. 1 already reported by Chanda & Owen (1951). Neither thyroxine nor thiouracil had any effect on the protein content of the fat-free milk.

Lactose, chloride and freezing point. These analyses, which were carried out in Exps. 1 and 2 only, show that, corresponding to a small increase in solids-not-fat (Table 2), there was a small increase in the lactose content of the milk, but the effect was not noticeable in all the treated cows. Thiouracil caused a small decrease in lactose content corresponding to a small decrease in solids-not-fat. Simultaneously with an increase in lactose content, there was a decrease in the chloride content of the milk in the thyroxine-treated cows. Thiouracil

between lactose and chloride it was also found that the freezing point of the milk remained unchanged throughout the experiments (Table 3).

Calcium, magnesium, sodium and potassium. The calcium content of the milk showed no change attributable to hormonal effects, thus confirming the earlier observations of Owen (1948*b*). In conformity with this finding is the observation that calcium was not affected by thiouracil. In Exp. 3, the mean calcium contents, with their standard errors, of the two thyroxine-treated cows were 136·7 ± 3·1, 130·8 ± 3·1 and 121·2 ± 3·0 mg./100 g. milk in periods 1, 2 and 3 respectively. The corresponding figures for the two cows which were treated with thiouracil were 124·4 ± 2·8, 118·8 ± 3·0 and 118·3 ± 2·8, and for the two control cows 121·2 ± 3·3,

$114{\cdot}8 \pm 3{\cdot}4$ and $110{\cdot}3 \pm 3{\cdot}1$. The failure of hormonal treatments to change the calcium content of milk is in sharp contrast to their effects on the phosphorus content which was increased by thyroxine (Owen, 1948*b*; Chanda & Owen, 1951) and decreased by thiouracil (Chanda & Owen, 1951). As pointed out earlier (Owen, 1948*b*), this can perhaps be related to the small amount of calcium required by the soft tissues. These observations, in conjunction with that of Owen (1948*b*), indicate that the metabolic pathways of calcium and phosphorus in adult animals are not so directly linked in the soft tissues as they are known to be in bone.

There was no indication that either drug caused any change in sodium, potassium or magnesium. The means and standard errors for all animals, without distinction of periods, were $64{\cdot}3 \pm 4{\cdot}8$, $149{\cdot}6 \pm 3{\cdot}6$ and $11{\cdot}3 \pm 0{\cdot}4$ mg./100 ml. milk for sodium, potassium and magnesium respectively.

Blood analysis. In view of reports that thiouracil treatment of toxic goitre in humans causes leucopaenia in a minority of patients (Grainger, Gregson & Pemberton, 1945; Himsworth, 1948), the blood of the cows in Exp. 3 was analysed for haemoglobin, red blood cells and white blood cells. The daily variations could not be related to the drug treatments, and there was no evidence of leucopaenia in the cows. The method of treatment in the present experiment was, however, by injection, whereas, in the treatment of toxic goitre in humans, thiouracil is given by mouth and much larger doses of the drug per unit body weight are used. Since leucopaenia caused by thiouracil is an idiosyncrasy it is conceivable that treatment of a larger number might demonstrate its occurrence in cows. We have, however, never observed any untoward symptoms in cows treated with thiouracil. The average figures for blood constituents of all the cows in Exp. 3 were: haemoglobin $8{\cdot}80 \pm 0{\cdot}17$ g./100 ml., red blood cells $5{\cdot}11 \pm 0{\cdot}14$ millions/mm.^{3}, and white blood cells $9{\cdot}31 \pm 0{\cdot}42$ thousands/mm.^{3}.

Creatine and creatinine content of milk. To find whether the increase in ester phosphorus caused by thyroxine (Chanda & Owen, 1951) could be attributed to an increase in labile phosphate, such as creatine phosphate, the creatine content of the milk was estimated in Exp. 3. The results showed that neither thyroxine nor thiouracil caused any change in the content of either creatine or creatinine. An analysis of variance showed that there was no significant variation in the creatine content of the milk between animals. There was, however, a significant decrease from period to period. This variation was attributable to advancing lactation since there was a regular trend of decrease during successive periods in all the cows. The averages

Table 3. *Effect of thyroxine and thiouracil on the lactose and chloride contents of milk*

Exp.	Cow	Daily injection in period 2	Period	Lactose (%)	Chloride (%)	Lactose chloride number*	Depression of freezing point (° C.)
1	1	None	1	4·13	0·151	6·17	0·545
			2	4·09	0·155	6·18	0·541
			3	4·05	0·158	6·18	0·542
	2	10 mg. thyroxine	1	4·22	0·145	6·18	0·546
			2	4·47	0·125	6·16	0·539
			3	4·16	0·152	6·21	0·544
	3	10 mg. thiouracil	1	4·33	0·137	6·18	0·538
			2	4·06	0·156	6·17	0·542
			3	4·37	0·139	6·25	0·546
2	4	None	1	4·39	0·132	6·17	0·542
			2	4·21	0·148	6·21	0·539
			3	4·09	0·155	6·18	0·541
	5	10 mg. thyroxine	1	4·67	0·112	6·18	0·545
			2	5·18	0·076	6·21	0·546
			3	4·76	0·105	6·18	0·542
	6	10 mg. thyroxine	1	4·28	0·144	6·22	0·539
			2	4·32	0·138	6·18	0·542
			3	4·11	0·154	6·19	0·537
	7	20 mg. thiouracil	1	4·25	0·142	6·17	0·547
			2	4·29	0·140	6·18	0·541
			3	4·35	0·139	6·23	0·545
	8	20 mg. thiouracil	1	4·31	0·138	6·17	0·551
			2	4·01	0·159	6·16	0·547
			3	4·44	0·135	6·26	0·549

* Lactose content + 13·5 × chloride content = 6·26 (Davies, 1936).

in periods 1–3 of creatine + creatinine (measured as creatinine) were $13{\cdot}7 \pm 0{\cdot}50$, $13{\cdot}1 \pm 0{\cdot}52$ and $11{\cdot}5 \pm 0{\cdot}62$ mg./100 g. in the thyroxine-treated cows, respectively. The corresponding figures for the cows which received thiouracil were $14{\cdot}0 \pm 0{\cdot}49$, $13{\cdot}2 \pm 0{\cdot}54$ and $11{\cdot}4 \pm 0{\cdot}62$, and for the control cows $12{\cdot}8 \pm 0{\cdot}50$, $13{\cdot}4 \pm 0{\cdot}50$ and $11{\cdot}8 \pm 0{\cdot}54$ mg./100 g. Creatine contributed a relatively constant percentage to these totals ($71{\cdot}2 \pm 4{\cdot}1$ %).

The lack of change in the creatine content of milk in thyroxine-treated cows is in sharp contrast to that of urinary creatine which was found to be markedly increased in cows treated with thyroxine (Owen, 1948*a*). The statement of Basu & Mukherjee (1943) that ester phosphorus in milk is exclusively the labile creatine phosphate is not supported by these results. In spite of the large increase in the ester phosphorus caused by thyroxine (Chanda & Owen, 1951), no changes occurred in the creatine content of the milk. Moreover, the amount of creatine found in the milk could have accounted for only 1·4 mg. ester phosphorus, which is lower than the smallest ester phosphorus figure observed by Chanda & Owen (1951) during thiouracil treatment.

Water-soluble vitamins

Ascorbic acid. The results for the five cows in Exp. 2 are recorded in Fig. 1 which shows that, in the thyroxine-treated cows, the ascorbic acid content of the milk decreased during treatment, while in the cows which were treated with thiouracil it increased. The control cow showed that the stage of lactation had little effect on the ascorbic acid content of the milk. In Exps. 1–3 the mean decrease caused by 10 mg. thyroxine in five cows was 25 % (range 14–34 %) while the mean increase caused in four cows by 20 mg. thiouracil was 24 %. In the cow dosed with 10 mg. thiouracil the decrease was 10 %.

Decreases in ascorbic acid content of milk after treatment of cows with iodinated casein have been recorded by Van Landingham, Henderson & Weakley (1944). Similar decreases were observed by Bartlett, Rowland & Thompson (1949) who attributed them to the iodide in the iodinated casein. Since, however, iodinated casein was not used in the present experiments, it is clear that it is the thyroxine itself which produces these effects. The minute dose of iodine given in 10 mg. thyroxine per day could not be expected to influence the ascorbic acid content of the milk. Furthermore, there is other evidence, albeit indirect, that the thyroid probably governs the ascorbic acid content of milk.

Riboflavin. The mean riboflavin contents of the milk of six cows (Exp. 3) in individual periods are recorded in Table 4 with their standard errors. The mean results for the two thyroxine-treated cows showed a decrease in the treatment period (period 2) compared with the initial control period (period 1), but this also occurred in one of the control cows and in the two thiouracil cows. The results were therefore analysed statistically. The analysis of variance, also recorded in Table 4, showed that the differences between cows were significant, but that the differences between periods within cows were not. The mean values in period 1 showed a range of 83–127 μg./100 ml. fat-free milk. It can, therefore, be concluded that neither the drugs nor the stage of lactation caused any change in the riboflavin content.

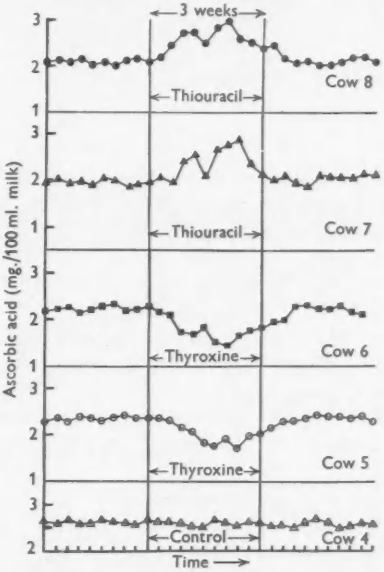

Fig. 1. Effect of thyroxine and thiouracil on the ascorbic acid content of cow's milk.

Bartlett *et al.* (1949) reported a 17 % decrease in riboflavin in the milk of cows treated with thyroxine. The Arizona workers (Kemmerer, Bolomey, Vavich & Davis, 1946) also reported a decrease, the concentration of riboflavin during the treatment period dropping almost to nil. These values, as Kon & Henry (1949) pointed out, are inconceivably low. In view of the finding of Bartlett *et al.* (1949) the present results of 2-day values were further analysed statistically to compare the rates of decrease in groups of cows during period 2. The differences between the three regressions were found not to be statistically significant. Kon & Henry (1949), quoting Thompson (1945), stated that neither thyroxine nor iodinated protein had any effect on the riboflavin content of milk.

Table 4. *Effect of thyroxine and thiouracil on the riboflavin content of cow's milk*

Cow	Daily injection in period 2	Riboflavin (μg./100 ml. fat-free milk ± S.E.) Period 1	Period 2	Period 3
9	None	103·2±1·6	105·4±2·2	102·2±4·2
10	None	82·5±1·7	77·2±2·9	77·0±2·4
11	10 mg. thyroxine	111·5±2·8	97·5±3·1	108·1±3·6
12	10 mg. thyroxine	103·0±1·5	95·1±2·9	100·2±3·4
13	20 mg. thiouracil	104·6±1·6	103·5±2·6	99·5±4·1
14	20 mg. thiouracil	127·2±2·2	115·7±2·5	107·0±4·2

Analysis of variance of the average results for riboflavin

(N.S., not significant.)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio (e^{2s})
Total	17	2687·58	—	—
Cows	5	2302·18	460·44	20·39*
Periods	2	159·61	79·81	3·53 (N.S.)
Error	10	225·79	22·58	—

* $P<0·001$.

Total aneurin and the partition of aneurin. The cocarboxylase and protein-bound aneurin in the milk studied in Exp. 3 are shown graphically in Fig. 2. In this diagram the individual 2-day data throughout the experiment are shown. The average values for total aneurin in each period are recorded in Table 5, where the phosphatase and the partition of aneurin between the free, the cocarboxylase and the protein-bound forms are also recorded. It can be seen from Table 5 that the total aneurin content of the milk gradually decreased in all the cows in successive periods. There were large variations in the total aneurin values between cows. An analysis of variance of the total aneurin showed that the differences between cows and between periods were statistically significant. Due to the significant difference between cows, comparisons cannot be made between the controls and the treated cows in the same period; and from a comparison of the same cow from period to period, the effect of the hormone treatment is difficult to establish, but it would appear that the normal rate of the decrease was slightly retarded by thyroxine and somewhat enhanced by thiouracil. In spite of the smallness of the change in the total aneurin, the partition of aneurin showed considerable change. Table 5 shows that under the influence of thyroxine the cocarboxylase in the milk increased markedly at the expense of the free aneurin. There was also a small increase in the protein-bound aneurin. These changes are well shown in Fig. 2. Decreases in the free form of aneurin in the milk of cows fed iodinated protein were reported earlier by Bartlett *et al.* (1949). The present experiments have shown that these decreases are accompanied by an increase in the cocarboxylase and in the protein-bound forms of aneurin when thyroxine is administered subcutaneously. At the peak of hormonal response, the cocarboxylase in the thyroxine cow no. 12, accounted for 38 % of the total aneurin compared with only 21 % in the pretreatment period. The present results showed that thiouracil increased the free aneurin content and decreased the contents of both

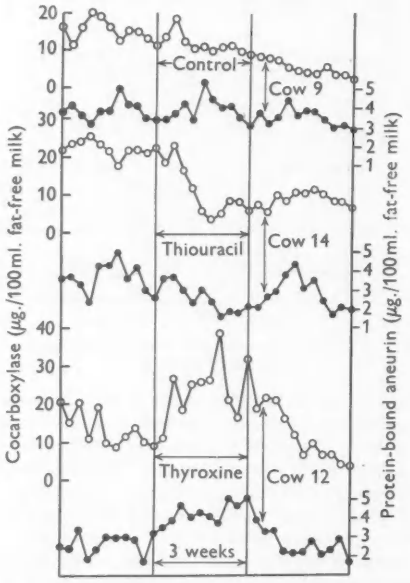

Fig. 2. Effect of thyroxine and thiouracil on the cocarboxylase and protein-bound aneurin content of cow's milk. ○—○, cocarboxylase; ●—●, protein-bound aneurin.

Table 5. *Partition of aneurin in fat-free milk*

Cow	Daily injection in period 2	Period	No. of analyses	Total aneurin (μg./100 ml.)	Percentage of total aneurin as: Free vitamin	Cocarboxylase	Protein-bound	Phosphatase (phenol units)*
9	None	1	11	39·4	50·4	38·0	7·3	59
		2	10	37·0	55·6	31·0	7·9	58
		3	11	37·1	72·7	14·9	6·9	109
10	None	1	11	46·5	48·0	39·9	7·6	47
		2	10	41·6	64·3	24·8	6·5	67
		3	11	40·5	70·6	18·5	6·6	78
11	10 mg. thyroxine	1	11	46·5	64·2	26·2	5·9	70
		2	10	46·0	30·7	58·2	7·3	35
		3	11	41·1	62·2	26·2	6·9	84
12	10 mg. thyroxine	1	11	42·0	58·1	31·5	6·7	67
		2	10	41·0	28·4	58·1	10·8	21
		3	11	39·2	56·9	30·9	7·0	73
13	20 mg. thiouracil	1	11	45·8	45·0	42·5	7·6	39
		2	10	39·1	68·4	21·1	7·3	70
		3	11	37·1	67·2	21·6	7·4	73
14	20 mg. thiouracil	1	11	50·3	42·7	44·6	7·4	37
		2	10	45·0	66·8	23·4	5·7	64
		3	11	44·3	68·3	20·5	6·4	59

* See Chanda & Owen (1951).

cocarboxylase and protein-bound aneurin (Fig. 2). The same sort of effect was noticed in the control cows (Table 5) as in the thiouracil cows, but it is evident from Fig. 2 and Table 5 that these natural effects were intensified by thiouracil. Thus, the minimum percentage of total aneurin in the form of cocarboxylase was decreased from 10 % in period 1, to 8 % in period 2 in the control cow (no. 10) while the corresponding decrease was from 18 to 4 % in the thiouracil-treated cow (no. 14).

During the last period of the experiment, the combined effect of advancing lactation and the cessation of thyroxine treatment produced some dramatic changes. The phosphatase titre increased rapidly and there was a big increase in free aneurin at the expense of cocarboxylase (Table 5). Protein-bound aneurin was also reduced. When thiouracil was discontinued, advancing lactation offset the expected sharp decrease in the phosphatase titre (Table 5) but protein-bound aneurin and cocarboxylase increased (Fig. 2). The effect of advancing lactation can be seen in the control animal (no. 9), in which, during period 3, a marked increase in phosphatase was accompanied by an increase in free aneurin and by corresponding decreases in cocarboxylase and protein-bound aneurin (Table 5). These effects were as unmistakable as those produced artificially by thiouracil in cows 13 and 14.

Correlation between phosphatase and phosphorylated aneurin. A positive correlation between phosphatase and free aneurin was observed to be a normal occurrence in the milk of cows and goats (Houston *et al.* 1940) and of sows (Braude *et al.* 1947) and also in human milk (Chanda, Owen & Cramond, 1951). This was confirmed in the present work.

There was a close positive correlation between phosphatase and free aneurin in the milk of all the cows (Table 6), and these correlations held whether the results for free aneurin were expressed in units per 100 ml. milk or as percentages of the total

Table 6. *Correlations of the percentages of aneurin in the free and cocarboxylase forms with phosphatase in the cow's milk*

(Number of pairs of observations was 28 in each case.)

Cow	Treatment	Coefficient* of correlation of phosphatase with: Free aneurin × 100 / Total aneurin	Cocarboxylase × 100 / Total aneurin
9	None	+0·9490	−0·8960
10	None	+0·8932	−0·8473
11	Thyroxine	+0·8794	−0·8898
12	Thyroxine	+0·9514	−0·9185
13	Thiouracil	+0·8419	−0·8257
14	Thiouracil	+0·8328	−0·7869

* Every coefficient shown in the table is significant at $P<0{\cdot}001$.

aneurin. There were also (Figs. 3 and 4) large negative correlations (Table 6) between phosphatase and cocarboxylase. The curves in Figs. 3 and 4 fitted by the method of least squares, show for a thyroxine and a thiouracil cow a curvilinear relationship between phosphatase and cocarboxylase similar to that found in human milk by Chanda *et al.* (1951).

In Fig. 5, in which mean values for phosphatase and for protein-bound aneurin are plotted, the regression line, fitted by the method of the least squares indicates a significant negative but non-linear correlation between the two variables.

Biochemical significance of the relation of phosphatase to phosphorylated aneurin. Like ester phosphorus, which was increased dramatically by thyroxine (Chanda & Owen, 1951), cocarboxylase and protein-bound aneurin were also markedly increased by the hormone. Thiouracil caused a significant decrease in cocarboxylase. Normally a higher proportion of phosphorylated aneurin is a characteristic of early lactation (Houston *et al.* 1940; Chanda, 1951). In late lactation the amount of phosphorylated aneurin is only 10% of the total aneurin so that the ratio of total to free aneurin approaches unity. The higher proportion of phosphorylated aneurin in early lactation and during thyroxine treatment may be related to the greater activity of the mammary gland. The phosphatase titre in milk becomes small, perhaps due to its retention in the gland at the peak of lactation or when thyroxine is administered to the cow. As the free aneurin in the milk of thyroxine-treated cows was observed to decrease with a concomitant decrease in the phosphatase, the contents of cocarboxylase and protein-bound aneurin were found to increase. The larger amount of phosphorylated aneurin appeared to occur at the expense of free aneurin because the total aneurin remained approximately constant. With thiouracil the proportion of free aneurin was increased at the expense of the phosphorylated fractions. When the mammary gland worked faster under the influence of thyroxine more of the phosphoric esters were synthesized (Chanda & Owen, 1951). This in turn was accompanied by an increase in the cocarboxylase. During hypothyroidism there is a lowering in the metabolic rate and consequently less of the milk aneurin is in the phosphorylated form. The lesser amount of phosphatase secreted in the milk during thyroxine treatment (Folley & White, 1936) may therefore denote a retention of the enzyme for synthetic activity in the gland. This hypothesis is supported by the close negative correlations observed between phosphatase and phosphorylated aneurin. The function of the milk phosphatase may thus be anabolic in the mammary tissue even though in milk after secretion it appears to be mainly hydrolytic (Graham & Kay, 1934). The interdependence of phosphorylated compounds and phosphatase may be related to the power of thyroxine to increase the concentration of enzymes in the body (Williams-Ashman, 1948). The reverse changes caused by thiouracil are also explicable on this basis. The negative correlations between phosphorylated compounds and phosphatase observed in this and in

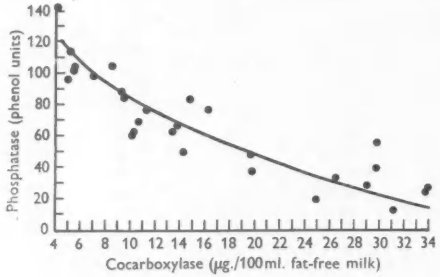

Fig. 3. Correlation between cocarboxylase and phosphatase in the milk of thyroxine-treated cow no. 11.

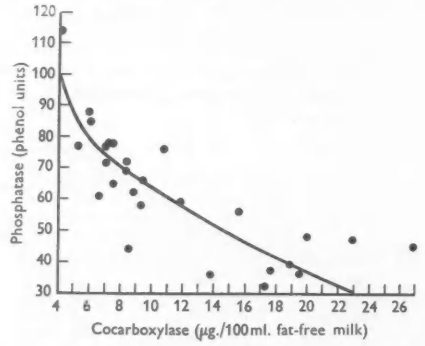

Fig. 4. Correlation between cocarboxylase and phosphatase in the milk of a thiouracil-treated cow.

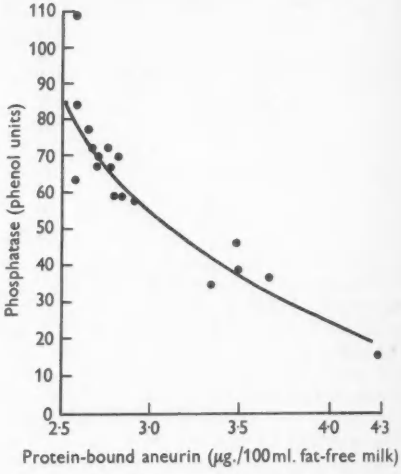

Fig. 5. Correlation between protein-bound aneurin and phosphatase in cow's milk. The ordinate of each point represents the mean value of phosphatase for a given cow in a given period, and its abscissa represents the corresponding mean value of protein-bound aneurin.

previous experiments (Chanda *et al.* 1951; Chanda & Owen, 1951) may imply a synthetic role for phosphatase in syntheses by the mammary gland.

SUMMARY

1. The effect of thyroxine and thiouracil on the composition of cow's milk has been investigated, and the relation of phosphatase to the partition of aneurin has been specially studied. The following are the main results:

2. Thiouracil (20 mg. per cow per day subcutaneously) decreased the milk yield of cows by 10·7 %, while DL-thyroxine (10 mg. per cow per day subcutaneously) increased it by 13·2 %.

3. Thyroxine caused an increase in the fat of milk in some of the cows and thiouracil caused a decrease.

4. There was a small increase in the lactose content of milk in the thyroxine-treated cows corresponding to a simultaneous decrease in the chloride content. Thiouracil caused the reverse effects. There was an inverse relationship between lactose and chloride, such that

lactose content = 6·19 − 13·5 × chloride content.

The freezing point of the milk was not changed by either drug.

5. The calcium, sodium, potassium, magnesium, protein, creatine and riboflavin contents of the milk were not demonstrably affected by either thyroxine or thiouracil. The creatine content of milk decreased with advancing lactation. Creatine phosphate was not found in the milk.

6. Thyroxine decreased the ascorbic acid content of the milk by 25 %, while thiouracil caused an increase of 24 %. When the dosage of thiouracil was halved, the increase was only 10 %.

7. Thyroxine did not affect the total aneurin content of the milk, but thiouracil caused a small but statistically significant decrease. Thyroxine increased markedly the cocarboxylase content of the milk, while a small but statistically significant increase also occurred in the protein-bound aneurin fraction. These increases were mostly at the expense of free aneurin which was decreased by thyroxine injection. Thiouracil changed the partition of aneurin in the opposite way. There were close negative correlations between phosphatase and cocarboxylase. Likewise there were close negative correlations between phosphatase and protein-bound aneurin. The correlations between phosphatase and free aneurin were positive. These correlations were found in all cows, irrespective of treatment with thyroxine or thiouracil. Implications of these correlations in the biochemistry of milk secretion have been discussed.

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Uptake of Potassium Tellurite by a Sensitive Strain of *Escherichia coli*

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As early as 1913 Schurmann & Hajos added potassium tellurite to a differential medium for the isolation of the diphtheria bacillus, and this use of potassium tellurite has now become familiar. Cavazutti (1921) and Joachimoglu (1920, 1922) found that in general Gram-positive organisms were less affected by tellurite than Gram-negative, and that the activity of related compounds decreased in the order $TeO_3^{2-} > SeO_3^{2-} > TeO_4^{2-} > SeO_4^{2-}$. Fleming (1932, 1942) demonstrated the value of potassium tellurite when used with penicillin and gentian violet for the isolation of certain bacteria from badly contaminated sources. Penicillin and potassium tellurite were both active at high dilution on entirely different bacterial species so that, with a few exceptions, bacteria which were penicillin-sensitive were tellurite-resistant and vice versa. Strains of *Escherichia coli* sensitive to tellurite could rapidly acquire resistance by serial subcultivation in increasing concentrations of the salt (Fleming & Young, 1940) and the antigenic and fermentation characteristics of both sensitive and naturally resistant strains of *Esch. coli* appeared to be identical. There was also some evidence of a very high absorption of tellurite by actively growing bacteria. Growth on tellurite agar yields black colonies, and granules can be seen inside the cells which are presumably composed of elementary tellurium (Hewitt, 1951).

In the present communication, as an approach to the means by which potassium tellurite (K_2TeO_3) inhibits bacterial growth, the mechanism of assimilation of potassium tellurite by bacterial suspensions is studied by the quantitative determination of the uptake using ^{127}Te incorporated into potassium tellurite as radioactive tracer.

Although the tellurium was added to the bacterial suspensions as the alkaline salt K_2TeO_3, the true substrate of the assimilation process at pH 5·5 may be TeO_2, H_2TeO_3 or $HTeO_3^-$. The pK_a values of the two dissociation stages of tellurous acid were found to be 8–9 and >10, so that only about 0·1 % of H_2TeO_3 is ionized at pH 5·5. Whatever the nature of this substrate, however, or of the final chemical form in which Te accumulates in the cells, uptakes and concentrations have been expressed throughout in terms of K_2TeO_3.

METHODS

Preparation of radioactive potassium tellurite

Acidic residues, containing radioactive Te obtained in the preparation of $^{131}I_2$, were supplied by the Atomic Energy Research Establishment, Harwell, with an activity of approximately 5 mc. in 100 ml. Oxalate, sulphate and chromic ions were also present, and elementary Te was slowly precipitated from the acid solution by the addition of 10 g. of $Na_2S_2O_5$ to 50 ml. of residues. After 48 hr. the precipitation appeared to be complete and the Te was separated and washed five times with distilled water. The moist Te was dissolved in a slight excess of 50 % (v/v) HNO_3, and the solution was cleared by centrifuging and diluted with distilled water to 6 ml. On addition of 20 ml. of ethanol a flocculent white precipitate of TeO_2 appeared. After standing overnight this was washed three times with distilled water in the centrifuge and dried for 24 hr. *in vacuo* over P_2O_5. The product was weighed, and the theoretical amount of 1 % (w/v) KOH solution added to form K_2TeO_3. The solution was finally diluted to a concentration of 1000 μg. K_2TeO_3/ml.

The overall yields of K_2TeO_3 were 100–150 mg., with an initial specific activity of about 0·5 μc./mg. and a radioactive recovery of 2 %. The half life of this solution corresponded exactly with that of ^{127}Te (90 days).

The concentration of the radioactive K_2TeO_3 solution was then confirmed by the colorimetric method of Shakhov (1945) and by isotope dilution with a standard 0·1% (w/v) K_2TeO_3 solution.

These determinations showed that the saturation concentration of K_2TeO_3 at 18° and pH 5·5 was 120–150 μg./ml. Accordingly, no experiments were conducted at a higher concentration than 100 μg./ml.

As a sample of commercial potassium tellurite was found to contain more than 30% excess of the theoretical quantity of TeO_2, the standard solution of K_2TeO_3 used above was prepared from dried TeO_2 obtained from the commercial salt by precipitation with acid.

Preparation of bacterial suspensions

One of the organisms most sensitive to tellurite among ten strains tested was found to be *Esch. coli* D. 433, from the National Collection of Type Cultures, and this has been used throughout these experiments. Saline suspensions were prepared from a 16 hr. agar slope, and used for inoculation of 250 ml. of the semi-synthetic medium described below. After incubation in Roux bottles at 37° for 16–18 hr., the cultures were harvested and washed three times with distilled water at 4°, yielding approx. 60 mg. dry wt. of bacteria/250 ml. medium.

1 l. of the medium contained 1 g. KH_2PO_4, 0·7 g. $MgSO_4.7H_2O$, 1 g. NaCl, 4 g. $(NH_4)_2HPO_4$, 0·5 g. trisodium citrate, and 0·5 mg. $FeCl_3$, together with 100 ml. of a neutralized acid casein hydrolysate containing 14·2 mg. N/ml. and 9·1 mg. PO_4^{3-}/ml., prepared by the method of Mueller (1939). NaOH solution was added to pH 6 and the medium was autoclaved in 250 ml. screw-capped bottles. The pH was unchanged by sterilization and growth.

Measurement of uptake

The procedure was similar to that previously used in this laboratory for the determination of the uptake of radioactive penicillin by bacteria (see Rowley, Cooper, Roberts & Smith, 1950). The washed bacterial suspensions, at approximately the required concentration, were aerated for 1 hr., as this procedure was found to increase tellurite uptake. A 0·1 ml. sample was diluted to 10 ml. for assay of bacterial dry wt./ml. on a calibrated Hilger absorptiometer. Samples (1 ml.) in centrifuge tubes were then incubated with buffer solution for 15 min. before addition of K_2TeO_3 and further buffer up to 5 ml. After a given time at 37° the tubes were cooled in ice-water, centrifuged rapidly (8 min. at 3500 *g*) and washed three times in distilled water after which the final supernatant solutions were entirely free of radioactivity. Care was taken not to remove bacteria with the supernatant solution. The washed bacteria were transferred to calibrated tubes and made up to 2 ml. with distilled water, and two 0·25 ml. samples were allowed to dry on planchettes for radioactive assay. As no attached radioactivity was found to be lost by the washing process, the uptake could then be calculated in terms of μg. of K_2TeO_3/mg. bacterial dry wt., knowing the specific activity of the K_2TeO_3 and the initial bacterial concentration.

Phosphate buffer (0·03 M) at the optimum pH (5·5) and temperature (37°) was employed, unless otherwise stated. Preliminary experiments were made to ensure that the total amount of tellurite removed from the solution did not decrease its concentration by more than 3%, which was regarded as an insignificant decrease as its effect was well within experimental error. This generally involved the use of bacterial concentrations <2 mg./ml. with tellurite concentrations of 40 μg./ml. and a reaction time of 45 min.

Radioactive assay

This was performed on the same apparatus used previously (Rowley *et al.* 1950). Total counting rates were generally of the order of 100/min., and duplicate planchettes were each counted for two 10 min. periods at different times during the day. The probable counting error is approx. ±2·0% for each sample. A background count (approx. 14/min.) was taken for 60–80 min. at midday and the value obtained was subtracted from the average total count for each sample. A uranium standard planchette was counted daily at three different voltages to determine plateau stability, together with a Te standard three times during the day. With Te no self-absorption correction was found to be necessary with the layer thicknesses used. Allowance was made for radioactive decay when calculating the weight of K_2TeO_3 bound by the cells.

The total error associated with the bacterial uptakes of radioactive Te was found to be approximately ±10%, composed of the counting error (±2%), the absorptiometer error of ±3% and the sampling error.

RESULTS

Non-removal of ^{127}Te *from* Escherichia coli *by washing with distilled water.* The amount of ^{127}Te attached to the organisms after incubation with radioactive K_2TeO_3, but before the organisms were washed, was calculated from the difference between radioactive assays of the bacterial suspension of known bacterial concentration and the supernatant solution after centrifuging. The supernatant solution was discarded and the bacteria resuspended to the same concentration as initially. The suspension was then re-assayed, centrifuged, and the supernatant re-assayed. This was repeated several times and the uptakes were found to be constant within experimental error.

The effect of temperature variation. The uptake of K_2TeO_3 by *Esch. coli* was found to be dependent upon the temperature at which the reaction between the bacterial suspensions and K_2TeO_3 occurred. Fig. 1 shows that the uptake was maximal at about 37°, falling off at higher or lower temperatures.

The activity of the bacteria in suspensions at different pH, *and in different buffers.* Fig. 2 shows the effect of variation of pH in the suspending medium on the uptake of K_2TeO_3.

The activity was maximal at pH 5·5 whether the sample was aerated beforehand or not, although aeration markedly increased the uptake by the organisms. A mixture of 0·03 M-acetate and 0·03 M-phosphate buffers was employed to ensure pH stability over the range 4·7–6·5. The use of 0·03 M-acetate, phosphate and succinate buffers gave approximately the same uptake at the optimum pH, but citrate buffer lowered the uptake by about

75%. Sodium citrate was also effective in preventing the precipitation of TeO_2 from K_2TeO_3 between pH 3 and 7 and it seems likely that a complex is formed between these salts.

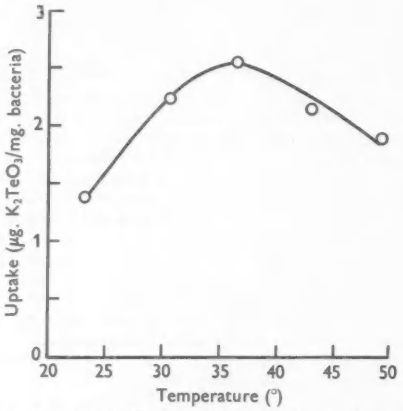

Fig. 1. Uptake of K_2TeO_3 on *Esch. coli* after incubation for 45 min. at different temperatures in 0·03 M-phosphate (pH 5·5) containing 40 μg./ml. K_2TeO_3.

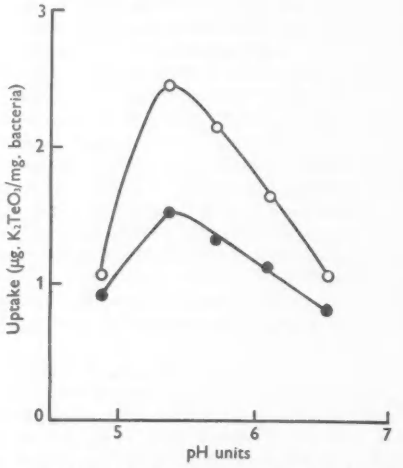

Fig. 2. Variation of uptake of K_2TeO_3 on *Esch. coli* with pH of the suspending medium. Organisms were incubated for 45 min. at 37° in a mixture of 0·03 M-phosphate and 0·03 M-acetate containing 40 μg./ml. K_2TeO_3. ○—○, one sample of bacteria aerated for 1 hr. at 18° before incubation; ●—●, the residue allowed to stand in contact with air at 18° concurrently.

In general, therefore, when experiments were performed at pH 5·5, 0·03 M-phosphate buffer was employed as this seemed without effect on the uptake.

Other factors affecting the activity of the system responsible for tellurite assimilation. The exposure of the bacterial suspensions to low pH for 1 hr. at 37° before adjusting to pH 5·5 caused a considerable decrease in the power of the organisms to assimilate K_2TeO_3 (Fig. 3). No loss of activity occurred at

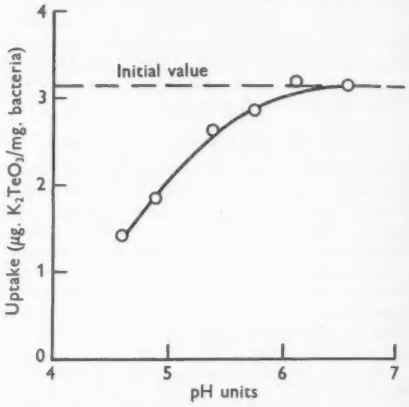

Fig. 3. Effect of pH on the stability of the system in *Esch. coli* responsible for tellurite uptake. Organisms were suspended in a mixture of 0·03 M-phosphate and 0·03 M-acetate for 1 hr. at 37°, centrifuged rapidly and resuspended in warm 0·03 M-phosphate at pH 5·5. K_2TeO_3 was then added to 40 μg./ml. and the uptake was assayed after a further 45 min. at 37°.

pH 6–7 and only about 10% at the working pH of 5·5. When the activity of the organisms was determined after different times of incubation at pH 5·5 there appeared to be a slight loss after several hours, but during the uptake period of 45 min. usually employed the loss was negligible (Table 1).

Table 1. *Rate of inactivation at* pH 5·5 *of the system responsible for the uptake of* K_2TeO_3 *by* Esch. coli

(K_2TeO_3 was added to a concentration of 40 μg./ml. after varying times of incubation at pH 5·5, and the radioactivity on the bacteria was assayed after a further 45 min. at 37°.)

Time (hr.)	Uptake (μg./mg.)
0	3·3
1	3·3
2·25	3·1
3·5	2·9
4·75	2·3
22	1·5

As bacteria contain easily oxidizable substances which interfere with processes such as methylene-blue reduction (Quastel & Whetham, 1925) it seemed advisable to determine the effect of removal of these substances before the addition of K_2TeO_3,

since this substance is also easily reduced. Accordingly, air was rapidly bubbled through the bacterial suspensions for 1 hr. at room temperature before commencing the experiment. In contrast with the effect on the rate at which methylene blue was reduced by bacteria, aeration was found to increase the tellurite uptake whilst treatment with nitrogen decreased it in relation to a suspension left in contact with air (Table 2). Treatment with nitrogen for 1 hr. after aeration gave uptake values which were identical with those of the aerated organisms.

Table 2. *Effect of aeration on the uptake of* K_2TeO_3

(Portions of an *Esch. coli* suspension at pH 5·5 were simultaneously stood in air, bubbled with air and bubbled with N_2 respectively for 1 hr. at room temperature. Half of the aerated sample was then bubbled with N_2 for a further hour, K_2TeO_3 was added to 40 μg./ml. to all four samples and the uptake was measured after a further 45 min. at 37° during which time the N_2 treatment was continued.)

Treatment	Uptake (μg./mg.)
N_2 bubbled	1·6
Stood in air	2·3
Air bubbled	2·8
Air bubbled for 1 hr. then N_2 for 1 hr.	2·7

The effect of the inclusion of glucose in the growth medium on the ability of Esch. coli *to assimilate tellurite.* Glucose was at first included in the growth medium in order to increase the yield of *Esch. coli*, but it was found that the resultant organisms had a lowered capacity to take up K_2TeO_3. Fig. 4 shows that as little as 0·05 % glucose markedly inhibited the formation of the tellurite-absorbing system so that the uptake was lowered whether the organisms were aerated before incubation with K_2TeO_3 or not. At this low glucose concentration the pH of the growth medium was scarcely altered after growth of the organisms so that the inhibition was not due to decomposition caused by fermentation acids before harvesting.

The further decrease in activity at higher glucose concentrations may be due to decomposition caused by acidity.

The effect of variation of the bacterial concentration on the uptake. When K_2TeO_3 at 40 μg./ml. was incubated for 45 min. at 37° with varying concentrations of *Esch. coli*, the uptake per mg. dry weight was found to be independent of the bacterial concentration, when this was less than 2 mg./ml. The uptake decreased at higher values, no doubt owing to the removal of a significant fraction of the K_2TeO_3 during uptake.

Variation of uptake with time of contact with tellurite. The amount of K_2TeO_3 attached to *Esch. coli* increased rapidly with time of incubation at 37°, reaching a maximum at 3–4 hr. and decreasing to a very low value after 22 hr. (Fig. 5). The rate of uptake was much less at 1°.

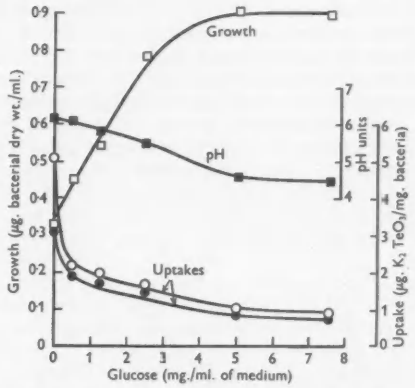

Fig. 4. Effect of inclusion of glucose in the growth medium on the ability of *Esch. coli* to take up K_2TeO_3, after being washed free from the medium. Organisms were incubated at 37° for 45 min. in 0·03 M-phosphate (pH 5·5) containing 40 μg./ml. K_2TeO_3. □—□, bacterial growth in the medium after 16 hr. incubation; ■—■, pH after growth (initial value 6·0); ○—○, uptake after prior aeration for 1 hr. at 18°; ●—●, uptake after standing for 1 hr. at 18° without aeration.

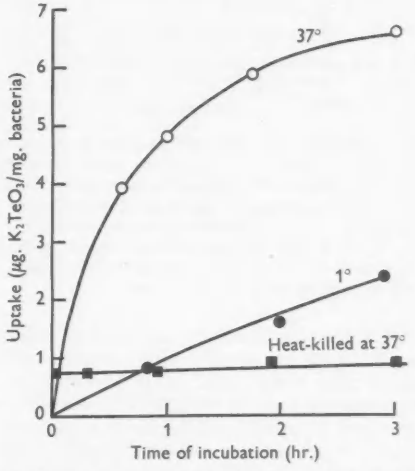

Fig. 5. Rate of uptake of K_2TeO_3 on aerated samples of *Esch. coli* in 0·03 M-phosphate containing 40 μg./ml. K_2TeO_3. ○—○, live organisms at 37°; ●—●, live organisms at 1°; ■—■, heat-killed organisms at 37°.

Variation of rate of uptake with concentration of tellurite. The amount of K_2TeO_3 attached to *Esch. coli* increased with the concentration of K_2TeO_3 in

solution in a manner shown in Fig. 6. When the reciprocal of the rate of uptake at 37° was plotted against the reciprocal of the tellurite concentration, following the procedure of Lineweaver & Burk (1934) for enzyme systems, a straight line resulted. Thus the relationship between the rate of uptake and the tellurite concentration was the same as that between the rate of an enzyme-catalysed reaction and its substrate concentration. The Michaelis constant (K_m) was calculated from this straight line by the method of Lineweaver & Burk.

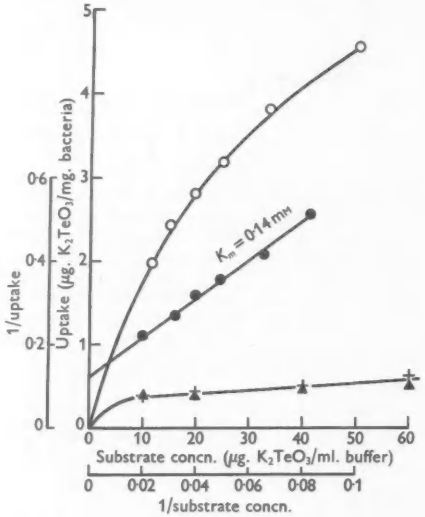

Fig. 6. Variation of uptake of K_2TeO_3 on *Esch. coli* with tellurite concentration. Organisms were incubated at 37° for 45 min. in 0·03 M-phosphate buffer (pH 5·5) containing K_2TeO_3. ▲—▲, heat-killed organisms unaerated; +—+, heat-killed organisms aerated for 1 hr.; ○—○, live organisms aerated for 1 hr.; ●—●, plot of reciprocal uptake against reciprocal tellurite concn. for the experiment using live organisms.

Table 3 shows that such experimental values of the Michaelis constant obtained varied between rather wide limits, but within these limits no difference was noticed whether the organisms were previously (*a*) grown in glucose, (*b*) unaerated, (*c*) aerated or (*d*) aerated and subsequently bubbled with nitrogen. Procedures (*a*) and (*b*) significantly decreased the activity of the organisms compared with the procedures (*c*) and (*d*).

The solubility of K_2TeO_3 at pH 5·5 was too low to allow the K_m to be derived by the alternative method of saturation of enzyme with substrate.

Effect of heat treatment. Immersion of a suspension of *Esch. coli* in a boiling-water bath for 20 min., followed by rapid cooling, altered the rate of uptake by the organisms (Fig. 5). A small amount of tellurite was taken up very rapidly, but after this no further uptake occurred. The amount of tellurite absorbed was also much less dependent upon concentration of the salt (Fig. 6), and the site of attachment appeared nearly saturated at quite low tellurite concentrations. Prior aeration had no effect.

Table 3. *Values for the Michaelis constant of the system responsible for* K_2TeO_3 *uptake on* Esch. coli

K_m (mM)			
Aerated organisms	Unaerated organisms	Aerated and bubbled with N_2	0·05% Glucose added to the growth medium, aerated
0·25	0·23	0·25	0·39
0·094	0·67	—	0·16
0·14	—	—	—
0·14	—	—	—
0·47	—	—	—

DISCUSSION

It has been shown above that potassium tellurite is rapidly assimilated by *Esch. coli* in amounts up to 1% of its dry weight and calculation shows that the concentration in the bacteria can reach 20 times the concentration in the medium. There is thus some mechanism allowing active transfer from the medium into the cells, and it appears very likely that this mechanism is associated with an enzyme or a group of enzymes. However, as Slater (1949) has pointed out, the fact that application of the Lineweaver & Burk equation gives a straight line is not conclusive evidence of an enzymic reaction, nor does this fact show whether or not an enzymic uptake has superimposed upon it an uptake of constant amount similar, for example, to that fixed by heat-killed organisms.

If the organisms were first killed by heat the uptake was radically altered, being much lower and much less dependent on K_2TeO_3 concentration, pH, time and temperature. The uptake curves in this case suggest a rapid chemical combination with the bacteria which is completed at relatively low tellurite concentrations.

The lowered activity when the organisms are grown with glucose suggests that the formation of the enzyme is inhibited in a similar manner to that reported by Epps & Gale (1942) for the formation of several enzymes in *Esch. coli* including certain deaminases and dehydrogenases. In these cases, as with the tellurite absorbing system, only a small part of the inhibition was due to the low pH in the medium induced by glucose.

As passage of nitrogen through the suspensions does not increase activity, the increase caused by aeration is unlikely to be due to the removal of volatile inhibitors. The activation is thus presumably due to the chemical action of oxygen and is not neutralized by the subsequent passage of nitrogen.

It is interesting that this aeration of the suspension before addition of K_2TeO_3 (a process employed by Quastel & Whetham (1925) to remove reducing substances before addition of methylene blue in Thunberg experiments) actually increased the rate of uptake of tellurite. The uptake of K_2TeO_3, which appears to involve a reduction to tellurium, may be similar to the reduction of methylene blue by bacteria observed in the absence of substrate in the Thunberg estimation. Both reactions use up hydrogen donors which are still present in the bacteria after washing and aeration, and one cause of the stimulation of tellurite uptake by oxygen may be due to the inhibition by this gas of alternative processes which utilize and remove the intracellular 'H' donors. This is similar to a suggestion made by Stephenson (1949) as an explanation of the Pasteur effect where yeast fermentation is repressed by oxygen in favour of complete oxidation of the substrates to carbon dioxide.

SUMMARY

1. By incorporating ^{127}Te into potassium tellurite (K_2TeO_3), the amount of the salt absorbed by a buffered suspension of a tellurite-sensitive strain of *Escherichia coli* could be calculated from a measure of the radioactivity remaining on the washed organisms. No radioactivity was lost by washing.

2. The system responsible for tellurite uptake was unstable at low pH but did not deteriorate greatly over several hours at pH 5·5–7.

3. The uptake of tellurite by the organisms increased with time, and was most rapid at pH 5·5 and 37°. The uptake had the properties of an enzyme action.

4. Growth of *Esch. coli* in a medium containing 0·05% glucose decreased the activity of the organisms, while the pH of the medium was scarcely affected.

5. Heating to 100° for 20 min. destroyed the enzymic system. The low uptake then obtained corresponded to a rapid combination with the cells which were saturated at fairly low tellurite concentrations.

Sincere thanks are due to Dr D. Rowley for his helpful suggestions. One of us (P.D.C.) is indebted to the University of London for a grant for apparatus from the Central Research Fund.

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Antibiotic Peptides from *Bacillus licheniformis*. Licheniformins A, B and C

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From the spore-forming aerobe *Bacillus licheniformis* Weigmann *emend.* Gibson, grown on an ammonium and sodium lactate medium, an antibiotic material, licheniformin, was prepared (Callow, Glover, Hart & Hills, 1947). It was obtained as the hydrochloride of basic material containing only carbon, hydrogen and nitrogen. It had bacteriostatic activity *in vitro* against numerous organisms, notably *Mycobacterium tuberculosis*, and protective effects against infections, including tuberculous infection, in mice. It was, however, toxic, causing damage to the kidneys after prolonged administration.

Further purification and chemical investigation was of importance from the practical point of view, to see whether the toxicity was inherent or could be separated from the antibiotic activity by fractionation. From the theoretical point of view, the constitution of a substance able to suppress experimental tuberculous infection was of the greatest interest. The crude active material for long resisted attempts at fractionation, but this was at last accomplished after it was found that separation into components could be effected by paper chromatography. This separation was used as a guide in devising a suitable scheme of countercurrent fractionation on a preparative scale.

The separation of three components, designated licheniformins A, B and C, is now described. They have proved to be peptides of very similar molecular weights and amino-acid composition, all having both antibacterial activity and toxicity, though to somewhat varying degrees.

Taken in conjunction with results obtained at the same time at two other centres of research, the Microbiological Research Department, Porton, and the Sir William Dunn Institute of Pathology, University of Oxford, the present results show that a single species of bacterium can produce at least six antibiotic polypeptides, one set of three, the licheniformins, on a medium with lactate and a high nitrogen/carbon ratio, and another set of three, the bacitracins, on a medium with glucose and a low nitrogen/carbon ratio. The late G. M. Hills collaborated not only with ourselves but also with the Oxford laboratories in devising suitable media for bulk production of antibiotic peptides from *B. licheniformis* and recognized the decisive influence of the medium on the type of antibiotic produced (Callow *et al.* 1947; Hart & Hills, 1947; Belton, Hills & Powell, 1949; Hills, Belton & Blatchley, 1949; Arriagada, Savage, Abraham, Heatley & Sharp, 1949).

EXPERIMENTAL

Materials and general methods

Organisms and culture conditions

We are indebted to the Microbiological Research Department, Porton, for culture fluids from the original strains of *B. licheniformis*, National Collection of Type Cultures (N.C.T.C.) no. 7072, and from the Oxford 'A 5', and to Boots Pure Drug Co. Ltd. for culture fluids from N.C.T.C. nos. 5399 and 6816, and from 'Plate 2C', another strain isolated by Dr P. D'Arcy Hart. Culture was on the medium previously described, or modifications of this (Callow *et al.* 1947; Hart & Hills, 1947; Belton *et al.* 1949).

Extraction of licheniformin

The processing of early batches was carried out as described previously (Callow *et al.* 1947). Later, however, after experiments with different adsorbents, it was found that Decalso F (Permutit Co. Ltd., London), a sodium aluminosilicate, acted as an adsorbent of high capacity from which licheniformin was readily eluted by NaCl solution. The procedure described below is more rapid and convenient than the active-charcoal process.

The supernatant from the autoclaved and centrifuged culture was brought to pH 5–6 and further clarified, if necessary, by filtration through a layer of Hyflo Supercel (Johns-Manville Co. Ltd., London) and then run on to a dry column containing 5 g. of Decalso F for each litre of liquid. A convenient column was provided by an inverted Winchester-quart bottle with the bottom removed and a bung and tap fitted in the neck. Fluid was fed from a large aspirator bottle, closed at the top and with a wide-bore tap and bent delivery tube from the lower tubulure dipping into the liquid above the column, so forming a constant-level device. The rate of flow was 0·3–1·0 l./hr. When the fluid level finally fell to the level of the top of the adsorbent 10% (w/v) NaCl solution was run on as eluent. The rate of flow was decreased to about 2 drops/sec. and the effluent was tested at intervals by adding 1 drop to 0·5 ml. of saturated aqueous solution of picric acid. When a definite precipitate was formed, collection of the eluate was begun and continued until the picric acid test became very weak. About 50 ml. of 10% NaCl was sufficient to elute the licheniformin

present in 1 l. of culture fluid of normal potency (500–1000 units/ml.). A slight excess of picric acid solution was added to the eluate, the precipitate was allowed to settle and collected on a sintered-glass filter (porosity 3). The picrate was decomposed on the filter by grinding with conc. HCl, the solution sucked through and run into a large excess of dry acetone with stirring. Crude licheniformin hydrochloride was precipitated. It was collected and dried *in vacuo* and further purified by solution in methanol (4 ml./g.), filtration, and reprecipitation by pouring into 25 vol. of acetone. The usual yield was 40–50 mg. of material with an activity of between 5 and 10×10^6 units/g. from 1 l. of culture fluid. With the rather inaccurate bio-assay method used, no significant loss of activity was detected during isolation.

Paper chromatography

Amino-acid separations were carried out as described by Dent (1948). The special solvent mixture for separation of licheniformin is described below.

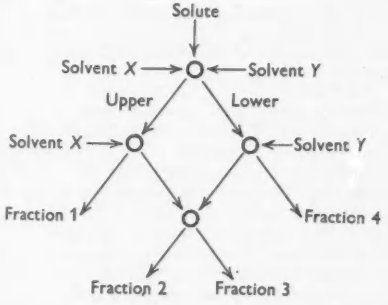
Antibacterial activity

This was estimated by the serial dilution method previously described (Callow *et al.* 1947) using *Mycobacterium phlei* as the test organism. We are indebted to Dr P. D'Arcy Hart for the numerous assays made in the course of this work.

Separation of licheniformins

Counter-current distribution between immiscible solvents

The system of fractionation described by Bush & Densen (1948) was used. In this *n* funnels, with final complete separation of the phases, yield $2n$ fractions. The system may be simply explained by means of a diagram showing the use of two funnels:



Bush & Densen (1948) give an equation which can be used to calculate the composition of the fractions in terms of the number of funnels used and the proportions of a substance in the upper and lower layers, p and q, respectively, where $p+q=1$. For a 12-funnel separation giving 24 fractions, these have the compositions: p^{12}, $12p^{12}q$, $78p^{12}q^2$, $364p^{12}q^3$, $1365p^{12}q^4$, $4368p^{12}q^5$, $12376p^{12}q^6$, $31824p^{12}q^7$, $75582p^{12}q^8$, $167960p^{12}q^9$, $352716p^{12}q^{10}$, $705432p^{12}q^{11}$, $705432p^{11}q^{12}$, $352716p^{10}q^{12}$, $167960p^9q^{12}$, $75582p^8q^{12}$, $31824p^7q^{12}$, $12376p^6q^{12}$, $4368p^5q^{12}$, $1365p^4q^{12}$, $364p^3q^{12}$, $78p^2q^{12}$, $12pq^{12}$, q^{12}. Using selected values of p and q a family of curves was drawn. By comparison of experimental graphs of weights of fractions against number in the series 1–24 it was then possible to estimate roughly the relative amounts and partition coefficients of major components of mixtures, by comparison of the heights and positions of the experimental maxima with the theoretical curves. The goodness of fit of a theoretical curve to the experimental curve for what appeared to be a single substance enabled one to judge its homogeneity with respect to partition coefficient. Bush & Densen also give an expression for the optimal proportions of upper and lower layers for separation of substances of known different partition coefficients. Thus, application of the procedure to separations on a preparative scale was simple over a range of partition coefficients, with appropriate alteration of the relative volumes of the two layers. At first ordinary separating funnels were used; later an automatic all-glass separating apparatus, as described by Craig & Post (1949), was constructed. This had 12 tubes each with a capacity of 92 ml. for the lower layer, and a maximum capacity of about the same for the top layer.

Paper chromatography

A large number of solvent mixtures were tried on one-dimensional paper partition chromatograms with descending flow. The only one effective in fractionating crude licheniformin was a mixture of collidine, lutidine and aqueous 2N-ammonia (1:1:2 by vol.). After vigorous shaking and separation, the upper phase was used in the trough and the lower used to saturate the atmosphere of the tank in which the chromatograms were run. Whatman no. 4 paper was used throughout. Spots were detected with ninhydrin spray in the way usual for amino-acids.

The procedure described below demonstrated the presence of three chromatographically distinct antibiotic components.

Licheniformin hydrochloride (1 mg.) was dissolved in water and transferred to four equally spaced spots at the top of a sheet of paper (length 50 cm.). The paper was irrigated with the upper layer of the solvent mixture. The solvent reached the end of the paper in 24 hr. and was allowed to run off the end of the sheet for another 24 hr. The paper was dried, washed thoroughly with ether until free of the odour of collidine and cut into four strips, each with one of the original spots at the top. Two of the strips were treated with ninhydrin and each was found to show several distinct zones of colour (Fig. 1). The four strips were laid side by side and the probable positions of the ninhydrin-positive spots marked in pencil on the untreated strips. The two untreated strips were now laid on the surface of two large agar plates (30×20 cm.) which had been previously poured with a uniformly distributed inoculum of licheniformin-sensitive bacteria, *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) or *Mycobact. phlei* (cf. Goodall & Levi, 1946). The plates were left at 0° for about 12 hr. and then incubated at 37° for 6 hr. (*Staph. aureus*) or 24 hr. (*Mycobact. phlei*). At the end of this time the paper was stripped from the surface of the agar and the plate examined for zones of inhibition (clear zones). The type of result which was obtained is shown in Fig. 1. The three clear zones on each agar plate corresponded with three coloured zones on the papers sprayed with ninhydrin. The three antibiotics separated by this procedure were named licheniformins A, B and C in order of increasing R_F values.

Unsuccessful attempts at fractionation

With paper-chromatographic analysis as a guide, various methods of larger-scale fractionation were tried. Fractional precipitation from aqueous solution by $(NH_4)_2SO_4$ or by methyl orange did not result in any separation. Activity was adsorbed by acid-washed alumina from methanolic solution, and material could then be eluted by aqueous methanol of increasing water content, but paper chromatography of the eluates showed that separation was far from complete. Elution of licheniformin adsorbed on Decalso F by dilute NaCl also gave only partial separation. Chromatography on a starch column in a mixture of collidine, lutidine and ammonia gave no evidence of fractionation. Ionophoresis in silica jelly (Consden, Gordon & Martin, 1946) promised interesting results, for under certain conditions three opalescent bands moved towards the cathode. It was, however, impossible completely to separate these bands, which tailed and became diffuse as they travelled, and little antibiotic activity could be recovered.

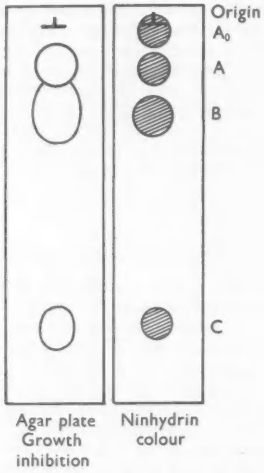


Fig. 1. Separation of three antibiotics by paper chromatography of crude licheniformin (for method see text). Zones of inhibition of bacterial growth are indicated by unshaded ovals. Zones coloured after spraying with ninhydrin are hatched.

Application of counter-current distribution

Several solvent systems were tried for the fractionation method outlined above. Licheniformin, as the hydrochloride, is stable but extremely soluble in water, and insoluble in the common organic solvents. Alkali metal hydroxides decompose licheniformin, but decomposition was not rapid in Sørensen borate-NaOH buffer at pH 12·38, and *n*-butanol extracted some activity from such a solution. *n*-Butanol and 8·5 N-NH_3 solution, *sec.*-butanol and 4·25 N-NH_3 solution and *n*-butanol, water, and piperidine (2:2:1 by vol.) all gave two liquid phases with licheniformin in each and were potentially useful for fractionation by partition, but they were unpleasant. Ultimately it was found that, in a mixture of phenol and water, a specimen of crude licheniformin had a partition coefficient (K) of 2·27 (ratio of concn. in upper water layer to concn. in lower layer); emulsification was largely prevented by addition of a little HCl. This system was used successfully.

The phenol-water mixture was prepared by melting the phenol ('detached crystals') and adding the appropriate amount of warm water. HCl (5 drops, about 0·25 ml., of 12 N/l.) was added, and the mixture equilibrated at room temperature. The crude licheniformin hydrochloride was dissolved in upper layer and the solution made up to the required volume and then added to the funnel containing lower layer; this constituted the 'first funnel' of the counter-current extraction. The final fractions were 12 water-rich upper layers and 12 phenol-rich lower layers. Recovery of material from these was at first done by way of the picrates, but this step was unnecessary and the following procedure was adopted, e.g. with 25 ml. upper layer and 75 ml. lower layer. Phenol was extracted from the upper layer with 80 ml. ether which was back-extracted with 2 × 20 ml. water. The combined water extracts were evaporated to dryness under reduced pressure; the residue was taken up in a little methanol and the solution poured into excess of acetone. The precipitated material was collected by centrifugation in a weighed 15 ml. tube, dried, and weighed. The lower layer was diluted with 20 ml. water and extracted with 200 ml. ether, which was back-extracted with 2 × 20 ml. water. The combined water extracts were then evaporated and material precipitated as before. Recovery was usually about 90% of the initial weight. Direct precipitation by pouring the layers into acetone was possible, but used much acetone.

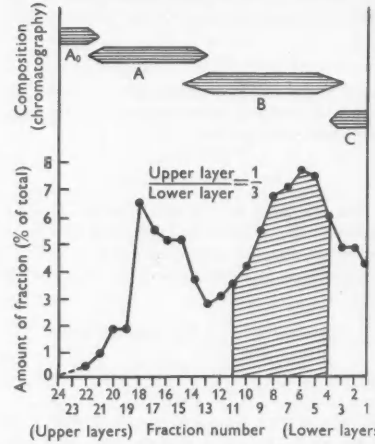

Composition (chromatography)
A_0
A
B
C
Upper layer = 1
Lower layer 3
Amount of fraction (% of total)
0 1 2 3 4 5 6 7 8
24 22 20 18 16 14 12 10 8 6 4 2
23 21 19 17 15 13 11 9 7 5 3 1
(Upper layers) Fraction number (Lower layers)

Fig. 2. Counter-current fractionation diagram for crude licheniformin hydrochloride partitioned between water (containing HCl) and phenol (for quantities see text). ▨ indicates material selected for refractionation to separate licheniformin B. The contents of each tube were also analysed by paper chromatography and the qualitative composition of the product is indicated diagrammatically above the curve.

The weighed fractions were examined by paper chromatography and a combined diagram could then be constructed in which weights and paper-chromatographic analyses were plotted against fraction numbers, as in Fig. 2, which gives results obtained with 2·14 g. of crude licheniformin hydrochloride prepared by the Decalso process from N.C.T.C. no. 5399 and partitioned between 12·5 ml. upper and 37·5 ml. lower layers (abbreviated as water/PhOH = 1/3).

The next step in the isolation of one of the constituents was to select fractions which were chromatographically homogeneous, or nearly so, with respect to the desired constituent. These were united and again submitted to counter-current fractionation. In doing this the ratio of upper and lower layers was altered so that the separation of the desired constituent from an adjacent one was more complete, e.g. alteration of the ratio water/PhOH to 1/0·75 brought constituent *B* into the upper layers with a maximum weight in fraction 18 and *C* was largely confined to the phenol layers. This is illustrated by curve *a* in Fig. 3, giving the results of taking fractions 4–11, wt. 0·92 g., from the previously illustrated fractionation (Fig. 2) and partitioning between 40 ml. upper and 30 ml. lower layer. A theoretical

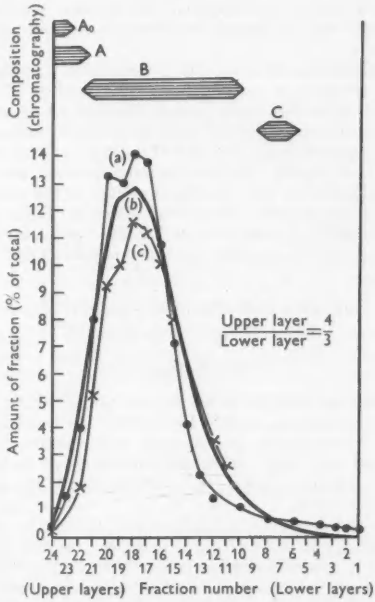

Composition (chromatography)
A₀
A
B
C
Amount of fraction (% of total)
(a)
(b)
(c)
Upper layer / Lower layer = 4/3
24 22 20 18 16 14 12 10 8 6 4 2
23 21 19 17 15 13 11 9 7 5 3 1
(Upper layers) Fraction number (Lower layers)

Fig. 3. (*a*) Counter-current fractionation diagram for partially purified licheniformin B hydrochloride partitioned between water (containing HCl) and phenol. The theoretical curve for $K = 1{\cdot}33$, $p = 0{\cdot}64$ with upper layer/lower layer = 4/3 is shown as a thick line (*b*). The qualitative compositions (paper chromatography) of the fractions are indicated diagrammatically above the curves. Curve *c* was obtained after two further counter-current fractionations of material represented by curve *a* (for details see text).

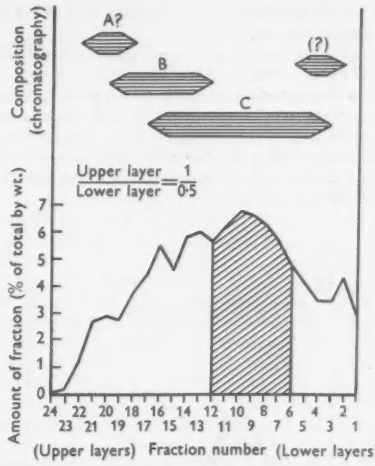

Composition (chromatography)
A?
B
(?)
C
Upper layer / Lower layer = 1/0·5
Amount of fraction (% of total by wt.)
24 22 20 18 16 14 12 10 8 6 4 2
23 21 19 17 15 13 11 9 7 5 3 1
(Upper layers) Fraction number (Lower layers)

Fig. 4. Counter-current fractionation diagram for crude licheniformin C hydrochloride partitioned between water (containing HCl) and phenol. ▨ indicates material selected for refractionation. The qualitative compositions of the fractions (paper chromatography) are indicated diagrammatically above the curve.

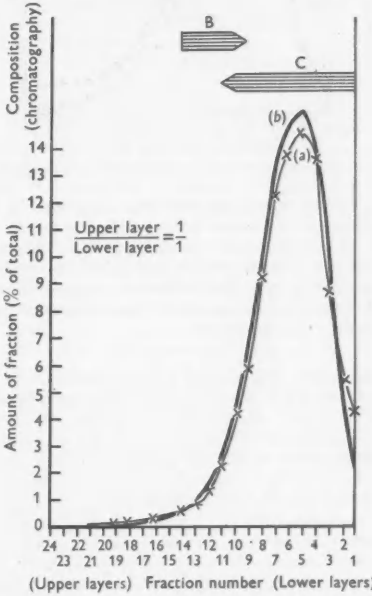

Composition (chromatography)
B
C
(b)
(a)
Upper layer / Lower layer = 1/1
Amount of fraction (% of total)
24 22 20 18 16 14 12 10 8 6 4 2
23 21 19 17 15 13 11 9 7 5 3 1
(Upper layers) Fraction number (Lower layers)

Fig. 5. Counter-current fractionation diagram for licheniformin C hydrochloride (*a*) partitioned between water (containing HCl) and phenol. A theoretical distribution curve (*b*) for $K = 0{\cdot}43$, $p = 0{\cdot}3$, is also shown. The qualitative compositions of the fractions are indicated diagrammatically above the curves.

curve, *b*, calculated on the assumption that $K = 1{\cdot}33$, is shown for comparison.

Generally, the procedure adopted was to select fractions rich in *B* from a number of different fractionations, unite them, fractionate with a ratio water/PhOH = 1/3 to remove the more water-soluble constituents, including *A*, to the upper layers, and then to unite the new fractions rich in *B* and conclude with a fractionation with a ratio water/PhOH = 4/3. The end result of one such fractionation is shown in curve *c* of Fig. 3.

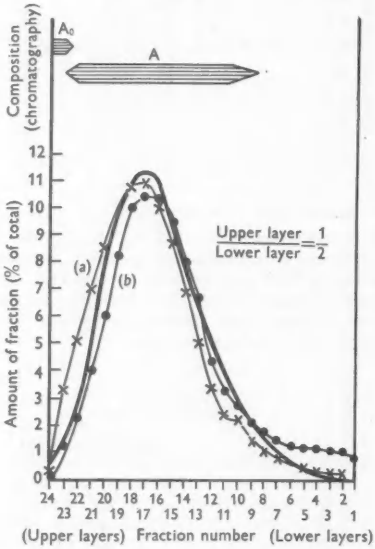

Composition (chromatography)
A_0
A
Amount of fraction (% of total)
Upper layer / Lower layer = 1/2
(a)
(b)
0 1 2 3 4 5 6 7 8 9 10 11 12
24 22 20 18 16 14 12 10 8 6 4 2
23 21 19 17 15 13 11 9 7 5 3 1
(Upper layers) Fraction number (Lower layers)

Fig. 6. Counter-current fractionation diagrams for two concentrates of licheniformin A hydrochloride partitioned between water (containing HCl) and phenol. A theoretical distribution curve for $K = 3{\cdot}0$, $p = 0{\cdot}6$ is also drawn (heavy line). The qualitative composition of the fractions (paper chromatography) is indicated diagrammatically above the curves.

Constituent *C* was isolated from a collection of *C*-rich fractions (wt. 3·8 g.) from various sources which, fractionated with a ratio water/PhOH = 1/0·5, gave the curve in Fig. 4. Fractions 6–12 from this (wt. 1·4 g.) were re-united and refractionated with a ratio water/PhOH = 1/1 with the result shown in Fig. 5, curve *a*. A theoretical curve *b* for $K = 0{\cdot}43$ has been drawn for comparison.

The same principles were applied to the separation of the constituent *A*. There was other material, A_0, more soluble in water, and remaining at or very near to the origin of the paper chromatograms, from which *A* had to be separated. A_0 was a brownish, deliquescent material and there was no evidence of segregation into a fraction of uniform partition coefficient. The counter-current fractionation diagrams of two concentrates of constituent *A* are shown in curves *a* and *b* of Fig. 6, together with a theoretical curve for material of $K = 3{\cdot}0$.

Licheniformin mixtures from different sources

By comparison of the counter-current fractionation diagrams of crude licheniformin from the five different strains of *B. licheniformis* worked up by active charcoal or Decalso, no evidence was obtained that there was any considerable difference in the proportions of the three main constituents. Charcoal preparations contained less *A* and *B* and more material highly soluble in phenol in comparison with Decalso preparations. Another constituent appeared on one occasion. In fractions 4–9 of a counter-current distribution at water/PhOH = 1/3 of a crude product from A 5, there was a constituent which moved slowly (at about the same rate as *a*) on a paper chromatogram. The amount of material available was not enough to encourage any attempt at isolation.

Some light was thrown on the composition of the material, often considerable in amount, at the extreme phenol-soluble end of the counter-current fractions by an experiment in which part of a lot of crude licheniformin was treated in 1% solution at pH 8–8·5 for 67 hr. at 37° with an equal weight of trypsin. The antibacterial potency was unaffected, and the only significant change in the counter-current diagram was a diminution of fraction 1 from 14% in the untreated material to 10% in the digested material. No effect on licheniformins A, B, or C was detectable.

Physical and chemical properties of licheniformins A, B and C

Physical properties

All three constituents in the form of hydrochlorides were white, amorphous, slightly hygroscopic powders, melting with decomposition at indefinite temperatures. Each behaved as a single substance when analysed by paper chromatography using the collidine/lutidine/ammonia solvent mixture.

Optical rotation. Crude mixtures had $[\alpha]_D$ − 36° to − 37·5° and $[\alpha]_{5461}$ − 42° to − 47° (*c*, 1) in water. Purified licheniformin A hydrochloride had $[\alpha]_D^{20°}$ − 37·4°; $[\alpha]_{5461}^{20°}$ − 45·2° in water (*c*, 0·19; *l*, 2 dm.). Licheniformin B hydrochloride had $[\alpha]_D^{20°}$ − 37·7°; $[\alpha]_{5461}^{20°}$ − 48·2° in water (*c*, 0·84; *l*, 4 dm.). Licheniformin C hydrochloride had $[\alpha]_D^{20°}$ − 36·8°; $[\alpha]_{5461}^{20°}$ − 44·9° in water (*c*, 1·18; *l*, 4 dm.).

Absorption spectra. The ultraviolet absorption spectra, measured on a Beckman spectrophotometer in aqueous solution, showed a rapidly increasing general absorption at decreasing wavelength below 2500 A. Bands corresponding to phenylalanine or inflexions in this region were observed with preparations of all three constituents, most clearly with licheniformin B, least clearly with licheniformin C, which had the higher general absorption. Selected curves are given in Fig. 7. No firm conclusions could be based on these absorption spectra, for, apart from generally absorbing impurities, there was some evidence in one fractionation of a material with a partition coefficient between those of licheniformins B and C and not visible on paper chromatograms and which had an intense band at 2480 A.

Molecular weight. We are indebted to Dr A. G. Ogston for determinations of molecular weights from sedimentation constants in the ultracentrifuge and diffusion constants. The values obtained were as follows: licheniformin A,

4400; licheniformin, B, 3800; licheniformin C, 4800. Full details are given in an Addendum to this paper (Addendum II).

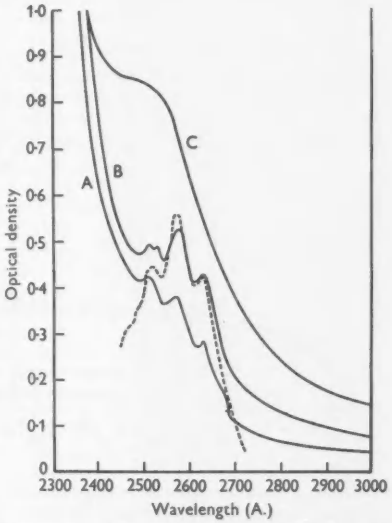


Fig. 7. Ultraviolet absorption spectra of licheniformins A, B and C and of phenylalanine (broken line). A and B were 0·2% (w/v) of hydrochloride in water, C was 0·1% (w/v) hydrochloride in water, and phenylalanine was 0·05% (w/v) in water.

Elementary analysis

The figures below are for material dried over P_2O_5 for 3–6 days.

Licheniformin	C (%)	H (%)	N (Dumas) (%)	Cl (%)
A	44·4	7·0	18·1	11·9
B (i)	44·7	7·2	18·4	10·4
B (ii)	46·2	7·1	17·7	11·3
C (i)	47·5	8·1	17·8	11·5
C (ii)	48·0	7·1	17·1, 16·4	—
C (iii)	47·5	7·7	18·1	—
C (iv)	45·2	7·0	17·0	10·5

(Analyses by Weiler & Strauss, Oxford, and by the Institute micro-analytical laboratory.)

Qualitative observation of peptide constitution

All three materials on acid hydrolysis gave amino-acids, evidently derived from an original peptide molecule. A sample of each antibiotic (250 μg.) was hydrolysed for 18 hr. with 6N-HCl at 105° in a sealed tube and after removal of acid the product was in each case analysed by two-dimensional paper partition chromatography. Licheniformins A and B gave chromatograms which indicated the presence of aspartic acid, serine, glycine, arginine, lysine, proline, valine and phenylalanine. Licheniformin C gave in addition a spot which was indistinguishable from glutamic acid.

Quantitative estimation of amino-acids in licheniformin: chemical method

Serine, glycine, proline, arginine and aspartic acid were estimated colorimetrically as their dinitrophenyl derivatives. A sample (40·3 mg.) of licheniformin A hydrochloride previously dried to constant weight at 100° was hydrolysed for 16 hr. in a sealed evacuated tube with excess 6N-HCl at 105°. The acid was removed and the residue dissolved in water and diluted to 4 ml. A sample of this solution (1·5 ml.) was allowed to react with an excess of fluorodinitrobenzene (FDNB) (0·15 ml.) in aqueous methanolic bicarbonate (6 ml. 5% (w/v) $NaHCO_3$, 6 ml. methanol) (Sanger, 1945) for 90 min. and the solution then acidified (10 ml. 3N-HCl).

From this point until all estimations were complete, operations were conducted entirely in a dimly lit room so as to avoid photodecomposition. The acid solution was extracted five times with 20 ml. peroxide-free ether, each extract being washed with 3 ml. water and the washings returned to the aqueous phase. Ether was removed from the aqueous phase under reduced pressure and the volume then adjusted to 50 ml. and 50 ml. of N-HCl added. Colour intensity was measured at once in a Hilger Spekker absorptiometer using a 1 cm. cell, tungsten lamp, violet filter (601) and heat filter (H 503). The amount of dinitrophenyl-(DNP-) arginine was determined by reference to a standard curve. The results are given in Table 1. Licheniformin B was treated in the same way. The combined ether extracts from the arginine determination were concentrated and the residue dried in a desiccator over KOH.

Use of Celite columns. Separation of the DNP-amino-acids was conducted on buffered Celite columns (Perrone, 1951). The Celite (Johns-Manville Co., London, S.W. 1), grade 545 (25 g.), was mixed with buffer (16 ml.) and peroxide-free wet ether (100 ml.) and stirred with a high-speed mechanical stirrer for 30 min. The suspension was poured into a suitable glass tube and the Celite packed with the packing tool described by Howard & Martin (1950). The diameter of the glass tube was chosen to give a final column about 15 cm. high. It was possible to separate from a single sample of 1·5 ml. of hydrolysed licheniformin hydrochloride (10 mg./ml.) the pure DNP derivatives of aspartic acid, serine, glycine, proline, valine and phenylalanine, but to do so it was necessary to use four successive columns of differing pH and the losses thus became serious. For quantitative estimation it was found that reproducible results could not be obtained unless the estimation of any one amino-acid was completed on a single column and within the compass of a single working day. The amino-acid which was being estimated was required to form a well defined band, separated from any other band, and the method of collection of the effluent was standardized both in this procedure and in the preparation of reference curves. When the band was 1 cm. from the bottom of the column the receiver was replaced by a measuring cylinder and when the trailing edge of the band left the column the volume of solvent in the cylinder was noted and a further 15 ml. of solvent collected before the receiver was changed. The solvent was evaporated as rapidly as possible *in vacuo*, the residue was dissolved in 1% $NaHCO_3$, the volume adjusted to 100 ml., and the colour intensity measured at once in an absorptiometer. By observing these precautions the variation between successive determinations of the same amino-acid was never greater than $\pm 5\%$.

Aspartic acid and serine. The Celite column was prepared in ether using buffer *A* of Blackburn (1949) and the ether then displaced by washed $CHCl_3$. The ether-soluble DNP-amino-acids from 1·5 ml. of licheniformin solution (10 mg. licheniformin HCl/ml.) were dissolved in the minimum volume of washed $CHCl_3$ and transferred to the column. The column was developed with $CHCl_3$ until the two slow bands (aspartic acid and serine) were well separated; the fast bands were discarded. In order to speed development the $CHCl_3$ was replaced by $CHCl_3$-butanol (99:1, v/v) and this solvent by $CHCl_3$-butanol (98:2, v/v). The serine band was collected first and the aspartic band then pushed through with $CHCl_3$-butanol (95:5, v/v). Estimations were made as described above. The results are given in Table 1.

Table 1. *Amino-acids in licheniformins* A *and* B

(Arginine, glycine, serine, proline and aspartic acid were estimated colorimetrically after conversion to their DNP derivatives and separation of the mixture on Celite. Phenylalanine, valine and lysine were estimated microbiologically.)

	Amino-acid (mg./100 mg. licheniformin)		N as % total N licheniformin	
	A	B	A	B
Aspartic acid	2·2	2·2	1·28	1·26
Glycine	11·9	11·8	12·3	11·9
Serine	6·5	6·9	4·8	5·0
Proline	5·5	5·5	3·7	3·6
Arginine	21·0	21·0	37·3	36·6
Phenylalanine	8·3	9·0	3·9	4·2
Valine	4·8	5·5	3·2	3·6
Lysine	36·6	38·6	38·6	40·0
Total	—	—	105·08	106·16

Glycine and proline. The procedure was the same as that used for aspartic acid and serine except that ether was used as solvent and the column was buffered with a mixture of 0·5 M-KH_2PO_4 (975 ml.) and 0·5 M-Na_2HPO_4 (25 ml.). The fast bands ($R \rightarrow 1$) were discarded and the next two bands (proline and glycine) collected. The results are given in Table 1.

Valine, lysine and phenylalanine. No column could be devised which would separate these three amino-acids as their DNP derivatives in a manner which eliminated all danger of error from overlapping bands and we accordingly abandoned the method in favour of microbiological assay.

Synthetic mixture. A mixture of glycine (25 mg.), serine (25 mg.), valine (25 mg.), phenylalanine (25 mg.), aspartic acid (25 mg.), arginine (75 mg.), and lysine (150 mg. monohydrochloride, $2H_2O$) in water was diluted to 15 ml. and samples of 0·5, 1 and 2 ml. used for preparation of the DNP derivatives. Three successive determinations were made of colour intensity for arginine, aspartic acid, serine, glycine and proline at each of these levels, the procedure outlined above being followed in each case. The results were highly reproducible, e.g. scale reading on absorptiometer: 0·475, 0·475, 0·485 for DNP-arginine from 2 ml. solution, and 0·25, 0·27, 0·26 for DNP-arginine from 1 ml. solution; 0·56, 0·58, 0·57 for proline from 2 ml. solution, and 0·38, 0·38, 0·39 for proline from 1 ml. solution.

Quantitative amino-acid analyses: microbiological methods (Miss K. R. de Bouk)

An account of these is given in an Addendum to this paper (p. 567).

D-*Amino-acids in licheniformins* A *and* B

A preparation of D-amino-acid oxidase was made from sheep kidney by the method of Negelein & Brömel (1939). Purification was carried only to the stage of the first $(NH_4)_2SO_4$ precipitation. Samples of the peptides were

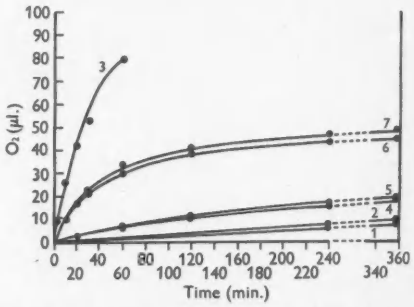


Fig. 8. Oxygen uptake (in µl. at 37°) during oxidation of hydrolysed licheniformin by D-amino-acid oxidase (for conditions see text). Curve 1, no substrate; curve 2, L-phenylalanine; curve 3, D-phenylalanine; curve 4, hydrolysed horse serum globulin; curve 5, hydrolysed casein; curve 6, licheniformin A; curve 7, licheniformin B.

hydrolysed for 8 and 16 hr. at 100° in 6 N-HCl in sealed, evacuated tubes. As controls, samples of casein and horse serum globulin were hydrolysed in the same way. The acid was removed from each sample by leaving in a desiccator over KOH for several days and the residue dissolved in water. The pH was adjusted to 8·0 by addition of 5% $NaHCO_3$ and the solution diluted to a concentration equivalent to 10 mg. peptide or protein/ml. The amount of D-amino-acid in each preparation was estimated by following O_2 uptake in Warburg manometers. Each flask contained 0·5 ml. hydrolysed peptide, 1·8 ml. sodium pyrophosphate buffer (pH 8·3, 0·067 M) and, in the centre cup, 0·2 ml. KOH (20%, w/v). The side arm of each Warburg flask contained 0·2 ml. enzyme. As controls three flasks were prepared, one with no substrate, one with D-phenylalanine (0·5 ml. ≡ 2·5 mg. amino-acid) and one with L-phenylalanine (0·5 ml. ≡ 2·5 mg. amino-acid). The result of a typical experiment is shown in Fig. 8.

Three successive runs were performed on separate samples of each peptide hydrolysed for 8 hr. and one on each peptide hydrolysed for 16 hr. The results were the same within the limits of error of the method. The two proteins hydrolysed for 8 hr. and for 16 hr. showed a slightly greater O_2 uptake than the control or the L-phenylalanine (10 µl. in 360 min.). After subtraction of the control value the corrected O_2-uptake figures for licheniformin A were 40, 37, 35, and 45 µl. O_2/5 mg. peptide hydrochloride, and for licheniformin B 45, 39, 35, and 45 µl. O_2/5 mg. peptide hydrochloride. These figures indicate that each peptide contained the same proportion of amino-acid in the D form. In the hope that the

D-amino-acid could be identified, the material from each Warburg flask was freed from enzyme (heat coagulation) and a portion was used for two-dimensional paper partition chromatography. Both peptides had initially given eight different amino-acids on hydrolysis and the same eight amino-acids were still present after oxidation with D-amino-acid oxidase. The conclusion is that in each peptide at least one species of amino-acid is present in both optical forms.

End amino groups in licheniformins A *and* B

The method of Sanger (1945) for the estimation of end groups using fluorodinitrobenzene was applied to each of the peptides. Each peptide (25 mg.) was treated with FDNB in the usual way and, after hydrolysis for 24 hr. at 105° with 10N-HCl (sealed tube), the product was separated into a water-soluble and an ethyl acetate-soluble fraction. The ethyl acetate-soluble fraction was passed through a column of silica gel and the very faint yellow band collected. Measurement of the colour intensity of this band in each case showed that it represented less than 6% of the theoretical value required for one end group. A sample of each DNP-peptide was also hydrolysed for 6 hr. with 6N-HCl, but no end group was found. Confirmation that both peptides had failed to react with FDNB was obtained by paper chromatography of the water-soluble fraction from the acid hydrolysis. This fraction contained ε-dinitrophenyllysine, and in addition, all the other amino-acids present in a simple acid hydrolysate of the peptide. The DNP-lysine was put on a column of silica with methyl ethyl ketone-ether (Sanger, 1945) as solvent. A single band indistinguishable from synthetic ε-DNP-lysine was obtained.

Biological tests

Antibacterial activity. The three antibiotics were tested *in vitro* against *Mycobact. phlei* and the greatest dilution capable of inhibiting surface growth in Hartley digest broth was recorded. Licheniformin A (hydrochloride) inhibited at 1 in 5×10^6; licheniformin B at 1 in 10×10^6; and licheniformin C at 1 in $0{\cdot}32 \times 10^6$. The figures quoted are averages for three successive tests.

Chronic toxicity (mouse). Table 2 shows the results of chronic toxicity tests for which we are indebted to Dr Janet Niven. The antibiotics were made up in sterile saline at about 5 mg./ml. and injected subcutaneously in mice. A sample of unfractionated licheniformin corresponding in properties with the licheniformin hydrochloride used in previous toxicity tests (Callow *et al.* 1947) was used for comparison and a sample of bacitracin was also tested. The assessment of kidney damage is subjective, but was always made by the same person and indicates the degree of micro-anatomical alteration in the kidneys of the experimental animals.

Mouse protection test against tuberculous infection. As licheniformin A was found by the chronic toxicity test to be much less toxic than B or C, it was tested further for its ability to control tuberculous infection in mice. The tests were made by the procedure outlined in an earlier paper (Callow *et al.* 1947) and streptomycin was simultaneously tested as a control. We are indebted to Dr P. D'Arcy Hart and Dr R. J. W. Rees for carrying out the tests. Licheniformin A was tested at two dose levels, 3 mg./day and 6 mg./day. The lower dose caused no obvious toxic symptoms but failed to control the infection; the higher dose controlled the infection to a lesser degree than streptomycin at the level of 0·3 mg./day and caused some kidney damage.

Table 2. *Chronic toxicity for mice of licheniformins* A, B *and* C, *compared with standard preparation and with bacitracin*

(Licheniformin A hydrochloride 5 mg./ml., licheniformin B hydrochloride 5 mg./ml., licheniformin C hydrochloride 7 mg./ml., bacitracin* 5 mg./ml., licheniformin standard (S 785) 5 mg./ml. Dose schedule 0·2 ml. morning, 0·4 ml. evening, and 0·4 ml. Saturday for 14 days (no dose given on Sundays). Each test made on three animals. The degree of kidney damage is indicated; +, slight; + + + +, severe.)

Licheniformin					
A	B	C	Bacitracin	S 785	Control (saline)
+	+ + + +	+ + +	+ +	+ + +	—
+	+ + +	+ + + +	+ + +	+ + +	—
+	+ + + +	+ + + +	+ + + + (died)	+ + +	—

* A specimen of commercially manufactured product provided by Dr F. L. Meleney.

DISCUSSION

A large number of antibiotics have been reported in the culture filtrates from aerobic spore-forming bacilli (Baron, 1950). The nature of the substance responsible for antibiotic action is not known in every case, but where a pure or nearly pure product has been isolated, activity has frequently been found in a peptide fraction (Gilliver, Holmes & Abraham, 1949; Sharp, Arriagada, Newton & Abraham, 1949; Howell, 1950; Catch, Jones & Wilkinson, 1949; Barry, Gregory & Craig, 1948; Consden, Gordon, Martin & Synge, 1947; Hotchkiss, 1944). In some instances an antibiotic peptide, which was at first thought to be homogeneous, has later been resolved into several distinct substances (Gregory & Craig, 1948).

In the present investigation, numerous attempts at purification of licheniformin hydrochloride by precipitation, salt formation and adsorption chromatography gave little or no evidence of heterogeneity, but paper partition chromatography showed that the best preparation of licheniformin previously obtained (Callow *et al.* 1947) was a mixture of at least three antibiotics. Counter-current distribution methods (Bush & Densen, 1948; Craig & Post, 1949) were successful in separating these three antibiotics, but nevertheless the purity of the final products, the hydrochlorides of licheniformins A, B and C, cannot be regarded as absolutely established. Each substance gave a single zone of colour with ninhydrin after paper chromatography and this zone coincided entirely with the zone of antibiotic activity. Each substance gave a distribution curve in a 24-stage counter-current fractionation which followed closely the theoretical curve for a pure compound. The materials are, therefore, each homogeneous with respect to partition coefficient between two pairs of solvents, but no crystalline derivative could be obtained.

We have been informed that Dr L. H. Kent, Dr B. T. Tozer and Dr J. H. R. Slade at the Microbiological Research Department have obtained very similar results with products from the A 5 strain on ammonium lactate medium. Like ourselves, they would put forward only with the greatest reserve the statement that any fractions are chemically homogeneous.

Biological activity

Crude licheniformin hydrochloride (Callow *et al.* 1947) had a wide antibacterial spectrum, but its main interest lay in its considerable activity against acid-fast organisms and in its potentialities as an antitubercular drug. The limitation to the use of the crude antibiotic was that it caused severe damage to the kidney. Purified licheniformin C was less active against acid-fast organisms than the original crude preparation and caused more extensive kidney damage. Licheniformin B was rather more active *in vitro* than the original preparation, but it also caused extensive kidney damage. Licheniformin A was much less toxic than either of the other fractions, but still caused some slight kidney damage and was less effective than streptomycin in controlling tuberculosis in mice (p. 565). It would be tempting to suppose that renal toxicity is not an inherent property of licheniformin A and that the small degree of kidney damage evident in our tests was due to contamination of this substance with another peptide but, since the criteria of purity were fairly exacting, we do not ourselves favour this view. Renal toxicity has been reported also with several other antibiotic peptides obtained from related bacteria, and attempts to separate this toxicity from the antibacterial activity have not been entirely successful (Miller, McDonald & Shock, 1950; Smith, Schultz, Ott & Payne, 1949; Abraham & Newton, 1950).

Chemical structure

The difficulty experienced in separating the three compounds indicated some similarity of composition, but we did not expect such close similarity as was actually found. Licheniformin C was readily distinguishable from the other two peptides in that it gave, on acid hydrolysis, glutamic acid in addition to the eight amino-acids found in licheniformins A and B. In other respects the three compounds showed remarkable similarity. Elementary analyses did not distinguish between them; all three had the same optical activity and about the same molecular weight; all three, after reaction with fluorodinitrobenzene, failed to give any 'end group' (Sanger, 1945) on acid hydrolysis.

Licheniformins A and B, being the more active antibiotics, were examined in greater detail than C. As can be seen from Table 1, the amounts of amino-acids produced on hydrolysis are strikingly similar: they account quite well for the whole of the original peptides and it seems unlikely that the difference in toxicity between A and B could be due to some unrecognized non-peptide component. The difference between the molecular weights of licheniformins A and B (4400 and 3800) is scarcely significant, having regard to the errors of the method, and in Table 3 the number of amino-acid residues/molecule of peptide is calculated on the assumption that the mean of these figures is the molecular weight of both peptides. There are three major sources of error in this calculation: (i) molecular weight, (ii) percentage composition as determined by micro-analyses and (iii) estimation of individual amino-acids by colorimetric or microbiological methods. Tristram (1950) has pointed out that in order to estimate the stoicheiometric ratio of amino-acids in a protein or peptide the percentage error in the analysis of any one amino-acid must not exceed $\pm 0{\cdot}4/R \times 100$, where R is the number of residues of that amino-acid. We have not had sufficient material to permit repeated quantitative analyses on our antibiotics, but we consider that the methods used cannot be relied upon to be more accurate than $\pm 5\,\%$, thus it may well be that the error approaches 10 % with a few of our amino-acid estimations.

Table 3. *Amino-acid residues in licheniformins* A *and* B

(Results are calculated assuming a molecular weight of 4100 for each peptide. Figures are given to nearest whole number but where there is doubt, the calculated analytical figure is given in brackets. For definition of permissible error, see text.)

	Licheniformin A	Licheniformin B	Permissible error (%)
Aspartic acid	1	1	40
Glycine	7 (7·4)	7 (7·2)	5
Serine	3	3	13
Proline	2	2	20
Arginine	6 (5·65)	6 (5·5)	6·7
Phenylalanine	2 (2·4)	2 (2·5)	13
Valine	2	2	20
Lysine	12	12	2·8

The last column of Table 3 gives the percentage accuracy required to calculate, with certainty, the number of residues of each amino-acid present in the unit of molecular weight 4100. It is apparent from these figures that our failure to find any significant difference in residue numbers between the two peptides does not necessarily prove that they have the same quantitative composition.

With these reservations in mind the close chemical resemblance between licheniformins A and B is

nevertheless quite striking and the difference in toxicity is difficult to explain. A difference in optical form of the constituent amino-acids of these two peptides is not attractive as an explanation, since each peptide, after hydrolysis, gave an amino-acid mixture in which the same proportion of amino-acid was present in the D form; however, the species of D-amino-acid was not established in either case. We are more inclined to suggest that the difference in toxicity between licheniformins A and B may be attributable to a difference in amino-acid sequence within the two peptides. Some attempts were made to confirm this idea by partial hydrolysis experiments, but both compounds gave mixtures of basic peptides which we could not fractionate.

The suggestion has already been made (Work, 1949) that certain antibiotics may act by interference with protein synthesis. It was pointed out that protein synthesis is a species-specific process and that inhibition of protein synthesis by metabolite analogues might be expected to be species-specific also. We have now obtained some evidence (Campbell & Work, 1951) that peptides are normal intermediates in protein synthesis and it is therefore not surprising that amino-acid sequence in an antibiotic peptide may influence toxicity. We have, as yet, no clue to the cause of the common nephrotoxic action of antibiotic peptides.

SUMMARY

1. The licheniformin of Callow *et al.* (1947) has been separated by the use of counter-current distribution into three antibiotic peptides, licheniformins A, B and C.

2. Licheniformins A and B are both hydrolysed by acid to give glycine, serine, proline, valine, phenylalanine, lysine, arginine and aspartic acid. Licheniformin C gives these amino-acids and, in addition, glutamic acid.

3. All three peptides have similar molecular weights (3800–4800), optical rotations and elementary compositions.

4. Licheniformins A and B are more active against *Mycobacterium phlei* than licheniformin C, and are less toxic to mice than licheniformin C.

5. Licheniformin A, the least toxic peptide, was less effective than streptomycin in controlling a tubercular infection in mice.

6. Quantitative amino-acid analyses were made on licheniformins A and B and the stoicheiometric ratio of the amino-acids calculated for each peptide.

7. Licheniformins A and B are both devoid of reactive end amino groups (fluorodinitrobenzene) and are presumably cyclic.

8. The difference in biological activity between licheniformins A and B is regarded as due to a difference in amino-acid sequence between the two peptides.

ADDENDUM I

Microbiological Estimation of Lysine, Valine and Phenylalanine in Licheniformin

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Although the estimation of lysine, valine and phenylalanine by microbiological methods is a standard procedure, some difficulty was experienced in using, unmodified, the media of earlier authors. After testing the media of Dunn, Shankman, Camien, Frankl & Rockland (1944), Barton-Wright (1946), Sauberlich & Baumann (1946) and Steele, Sauberlich, Reynolds & Baumann (1949), some modifications were made to the last two, and good acid production with linearity over a substantial part of the curve was attained. A brief account of these modifications is given. The results obtained are given in Table 1 (p. 564).

MATERIALS AND METHODS

Cultures. Lactobacillus arabinosus 17–5 was used for the determination of valine and phenylalanine; *Leuconostoc mesenteroides* P–60 was used for the determination of lysine.

Stock stab cultures were carried in a Nymon & Gortner (1946) medium, modified as follows. For 100 ml. of medium: Difco bacto-peptone, tryptone and yeast extract, 0·5 g. each; liver extract, 10 ml.; glucose 1·0 g.; sodium acetate (hydrated), 1·0 g.; 0·5 ml. each of salt solutions *A* and *B* (see basal medium); agar, 1·5 g. This medium maintained vigorous growth for all the organisms commonly used for amino-acid assays (*Lb. arabinosus* 17–5, *Lb. mesenteroides* P–60, *Streptococcus faecalis* R, *Lactobacillus fermenti* 36

Leuconostoc citrovorum, *Lactobacillus brevis*), and was an improvement on published media on which cultures tended to die out. Transfers were made monthly. This practice was discontinued, however, in the case of *Lb. arabinosus* owing to a gradual departure in linearity of response and the latter was maintained in the dried state. Before use in assays the organisms were grown in broth of the same composition as the above, with the agar omitted.

Basal medium. Medium I of Sauberlich & Baumann (1946) was used with *Lb. arabinosus* 17–5 with the following modifications. Serine, glycine and proline were omitted from the medium as unnecessary; sodium acetate (hydrated) was increased from 20 to 33 g./500 ml. of double-strength medium; 200 mg. of L-asparagine was replaced by 640 mg. of DL-aspartic acid; riboflavin was reduced from 0·5 to 0·25 mg. (see below).

Medium VI of Steele *et al.* (1949) used with *L. mesenteroides* P–60 was modified by increasing the vitamins. 500 ml. of double-strength medium contained: aneurin 1·0 mg.; pyridoxin, 1·6 mg.; calcium D-pantothenate, 1·0 mg.; nicotinic acid, 2·0 mg.; *p*-aminobenzoic acid, 0·1 mg.; biotin, 0·005 mg.; folic acid, 0·01 mg.; riboflavin, 0·25 mg. The smaller quantity of riboflavin used in both media I and VI served to diminish the colour of the medium and hence to facilitate titration, and in no way affected the assays.

The inorganic salt solutions used to make up 500 ml. of media (double strength) were as follows: Solution *A*, 500 mg. of KH_2PO_4 and 500 mg. of K_2HPO_4 in 5 ml. Solution *B*, 200 mg. of $MgSO_4.7H_2O$, 10 mg. of $FeSO_4.7H_2O$, 20 mg. of $MnSO_4.4H_2O$, and 10 mg. of NaCl in 5 ml.

Standard curve. The synthetic DL-acid was used, after confirming that the D-form was not available with the media employed; however, calculations were based on the L-isomers only. The range (per tube) for the construction of the linear portion of the curve was as follows: L-lysine HCl, 0–50 μg., L-valine and L-phenylalanine, 0–25 μg.

Preparation of samples for assay. Licheniformin hydrochloride (15 mg.) was hydrolysed in 6N-HCl for 16 hr. at 105° in a sealed evacuated tube. The HCl was removed over KOH and the residue made up to 10 ml. in water. Portions taken were adjusted to pH 6·8.

Assay procedure. The sample was added to 5 ml. quantities of double-strength medium and the tubes sterilized at 10 lb./in.^{2} for 5 min. This gave less darkening of the medium than with the longer periods and higher pressures commonly used. The lactic acid was titrated to pH 6·8 with 0·1N-NaOH using Smith's (1930) indicator suitably altered so as to produce the colour change of orange-grey to pure grey at pH 6·8 instead of 6·9. This modified indicator, containing 0·09 g. of bromothymol blue and 0·10 g. of alizarin red sulphonate dissolved in 100 ml. of 30% (v/v) aqueous ethanol, gives a much sharper end point than the bromothymol blue always recommended in the literature for microbiological assay titrations.

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ADDENDUM II

Sedimentation and Diffusion of Licheniformins A, B and C

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Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge by the method of Cecil & Ogston (1948). The hydrochlorides were dissolved in 0·2 M-NaCl to a concentration of 1 g./100 ml. Owing to the relatively small molecular weights and high diffusion coefficients, clear sedimentation boundaries were not obtained; the sedimentation constants were therefore measured by the method of Gutfreund & Ogston (1949). These results are probably not more accurate than ±5%.

Diffusion. This was measured in the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948). The same solutions were used as for sedimentation. Since an equilibrium diffusate could not be prepared by dialysis, the solvent into which diffusion took place was NaCl of a concentration calculated to equal that of diffusate on the basis of the Gibbs-Donnan equilibrium. The observed diffusion coefficients are then comparable with the sedimentation constants. Both sedimentation and diffusion constants are corrected to their values in water at 20° (Table 1). In calculating the molecular weights, a value of 0·75 has been assumed for the partial specific volume.

Table 1. *Sedimentation and diffusion constants*

Licheniformin	S_{20} (corr.) × 10^{13}	D_{20} (corr.) × 10^{7}	Mol.wt.
A	0·96	21·0	4400
B	0·86	22·4	3800
C	0·95	19·4	4800

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A Solubility Analysis of Crystalline Ox-Liver Catalase

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Loew (1901) first demonstrated that the decomposition of hydrogen peroxide by plant and animal tissues was due to an enzyme which he named catalase. Other workers proceeded to isolate and purify this enzyme and eventually Zeile & Hellström (1930) established that the enzyme was an iron-porphyrin protein. This was confirmed by Keilin & Hartree (1936) and Stern (1935), who also proved that the iron-porphyrin was protohaematin. Sumner & Dounce (1937) crystallized ox-liver catalase soon afterwards; since then many other liver and erythrocyte catalases and a bacterial catalase have also been prepared in a crystalline state.

Catalase has been found to be present in all aerobic cells so far examined and is present in high concentrations of from 10^{-6} to 10^{-7} M in the liver, kidneys and erythrocytes of mammals. Dounce (1943) has stated that the catalase of liver is confined to the cytoplasm of liver cells, but has since found (1951) that this observation was due to the method of preparation of the liver-cell nuclei and that nuclei prepared by an improved method do contain catalase.

Stern & Wyckoff (1938), using an ultracentrifuge method, found the molecular weight of ox- and horse-liver catalases to be about 250000, and from the iron content of their preparations calculated that the catalase molecule contained four atoms of iron. Sumner & Dounce (1937) observed that only about half the iron of their crystalline ox-liver catalase preparations was bound as haematin, and Lemberg, Norrie & Legge (1939) later demonstrated

that part of the iron of liver catalases is bound in the form of a catalytically inactive bile-pigment haematin. Bonnichsen (1948) has put forward evidence which indicates that the bile-pigment content of liver catalases may be due to the methods of preparation.

The fractional values for the molecular ratio of haematin and catalase protein and the variations of specific activity obtained with different preparations, has prompted Sumner (1941) to suggest that liver-catalase preparations contain a mixture of catalases with one, two, three and four intact haematin molecules per catalase molecule, the remaining iron being bound as inactive bile-pigment haematin. According to Lemberg & Legge (1949), the values of specific activity or catalase capability (*Kat.f.* defined by von Euler & Josephson, 1927) for most purified preparations of liver catalases described in the literature vary between 52000 and 60000, when corrected to zero bile-pigment content. Agner (1942), however, has reported a *Kat.f.* value of 80000 for a horse-liver catalase preparation, when corrected to zero bile-pigment content. This value has since been confirmed by Agner & Theorell (1946).

In a preliminary study of the Sumner & Dounce method of preparing ox-liver catalase, the author found that homogenizing the minced tissue in a Waring Blendor increased the measured *Kat.f.* of the crystalline catalase from 45000 to 72000. The present paper describes an analysis of this highly active preparation using a solubility test method designed to detect and characterize the components of the suspected mixture of catalases. It is shown that the increase in *Kat.f.* of the final product after homogenizing the tissue is due to the presence of a small amount of a catalase with a much higher *Kat.f.* than hitherto observed, and some of the optical properties of this new catalase have been measured.

EXPERIMENTAL

Preparation of enzyme

Crystalline ox-liver catalase was prepared by a modification of the method of Sumner & Dounce (1937) as follows:

Ox liver (2 kg.) was minced, homogenized for 2 min. at 0° in a Waring Blendor, extracted with 35% (v/v) aqueous dioxan and fractionated with dioxan at 0° according to Sumner & Dounce. The precipitated fraction, which contained the catalase, was extracted three times with a dilute solution of salivary amylase in distilled water (total vol. 25 ml.) to digest the glycogen, and the catalase isolated from this extraction solution in a crystalline form by seeding and allowing to stand at 0° for 24 hr. The crystals were centrifuged down at 0°, dissolved in 0·1 M-phosphate buffer, pH 7·4, and recrystallized by dialysing against 0·1 M-phosphate buffer, pH 6·3. These crystals were centrifuged at 0°, dissolved in 0·1 M-phosphate buffer, pH 7·4, and dialysed against 0·1 M-phosphate buffer, pH 6·55. The mother liquors from the two crystallizations were also dialysed against this buffer for 2 days before carrying out the solubility test.

Solubility test method

Amounts of $(NH_4)_2SO_4$ calculated to produce a suitable range of ionic strengths were dissolved in 5 ml. samples of the enzyme solutions containing about 1 mg. protein/ml. After allowing to equilibrate at 0·5° for 24 hr. the solutions were filtered at the same temperature, as described by Falconer & Taylor (1946*a*). The optical densities of the filtrates at 275, 405 and 502 mμ. the maxima of the catalase absorption spectrum, were measured in a Beckman spectrophotometer. This procedure was carried out with the twice-crystallized material and with the two mother liquors. The *Kat.f.*, of the twice-crystallized catalase, was determined by the method of von Euler & Josephson (1927) and the relative catalase activities of the filtrates determined by the perborate method of Feinstein (1949) at 10°. The protein N content of some of the filtrates, selected on the basis of the salting-out curves, were estimated by the micro-Kjeldahl method of Falconer & Taylor (1946*a*). Total haematin content was estimated by the method of Duve (1949).

RESULTS

The salting-out curves for the twice-crystallized material in terms of the variations of optical densities at 275, 405 and 502 mμ. with ionic strength are shown in Fig. 1*a–c* respectively, and the corresponding changes in enzyme activity are shown in Fig. 1*d*. The specific property solubility diagrams of Falconer & Taylor (1946*b*), shown in Fig. 1*e*, *f*, clearly demonstrate the complex nature of the preparation. It is apparent from Fig. 1 that there are three main components *A*, *B* and *C* with appreciable absorption coefficients at 405 mμ., together with a small amount of material that absorbs at 275 mμ. but not at 405 mμ. and remains in solution at an ionic strength of 6·5. An analysis of the constitution of the preparation and of the nature of the components, derived from the solubility curves and from the protein-nitrogen estimations, is presented in Table 1.

Protein *C* has a *Kat.f.* value of 45000 and a ratio of the optical density at 405 mμ. to that at 275 mμ. of 0·9 and therefore corresponds to the catalase usually obtained from ox liver by the unmodified Sumner & Dounce method. From the complexity of the sections of the graphs in Fig. 1*e*, *f*, corresponding to the salting-out of this component, it is possible that *C* is a mixture of catalases differing only in the number of intact haematins per catalase molecule. Protein *A*, with a *Kat.f.* value of 180000, has not been observed before, and its presence accounts for the increase in *Kat.f.* to 72000, when the usual method is modified by the introduction of the blending process. The *B* component is apparently a haem protein with no appreciable catalase activity.

The observation of the optical densities of those filtrates of the two mother liquors with ionic strengths between 3·5 and 5·2 was prevented by the following interesting effect. Immediately a portion of these filtrates was exposed to radiation of wavelengths 275, 405 or 502 mμ. issuing from the monochromator of the Beckman spectrophotometer, the clear solution became cloudy due to precipitation. After placing the samples in darkness for 1 hr. the cloudiness disappeared, but reappeared again on exposure to the radiation from the Beckman or to ordinary daylight. No appreciable change in temperature occurred in the solution during this precipitation process, and the effect could not be produced by raising the temperature of the samples to 30°. Fig. 2, which shows the salting-out curve of the mother liquor from the second crystallization, indicates that a component (*X*) is being salted out

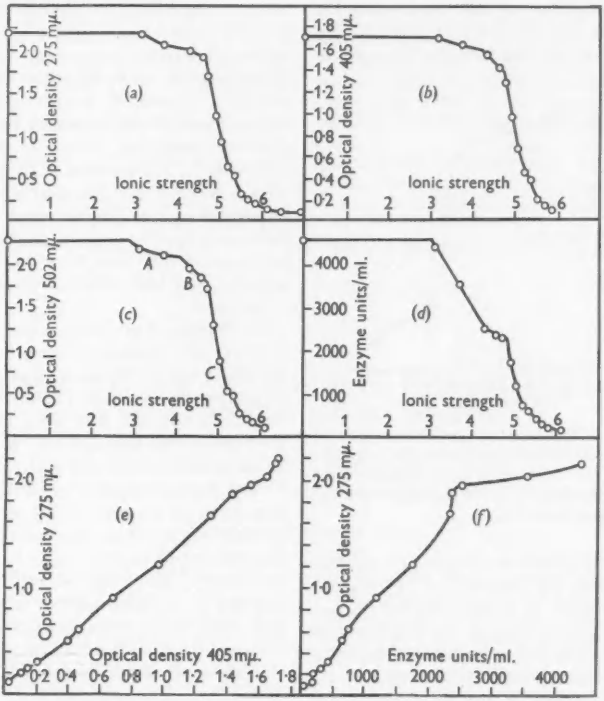

Fig. 1. Solubility test curves of twice-crystallized ox-liver catalase at pH 6·55 using solid $(NH_4)_2SO_4$ as salting-out agent. (*a*), (*b*) and (*c*) are the precipitation curves for proteins in solution with absorption at 275, 405 and 502 mμ. respectively; (*d*) is the precipitation curve for catalases in solution, and (*e*) and (*f*) are the 'specific property solubility curves' for the solution.

Table 1. *Solubility test analysis of twice-crystallized ox-liver catalase constitution of original solution*

(*Kat.f.* = 72000; moles haematin/moles catalase = 3·2; optical density 275 mμ./optical density 405 mμ. = 1·3; % component *A* = 17; % component *B* = 4; % component *C* = 78.)

	Properties of the components		
	A	*B*	*C*
Enzyme activity/optical density 275 mμ.	6800	0	1810
Enzyme activity/optical density 405 mμ.	15490	0	1630
Optical density 405 mμ./optical density 275 mμ.	0·43	1·3	0·90
Optical density 502 mμ./optical density 275 mμ.	0·048	0·13	0·11
Optical density 502 mμ./optical density 405 mμ.	0·11	0·10	0·12
Kat.f.	180000	0	45000

over the range of ionic strengths at which the 'photo-precipitation' effect occurs and therefore that component *X* is in equilibrium with its solvent in the filtrates with ionic strengths in this range. It seems clear, then, that the precipitation effect is due to a decrease in solubility of this component on absorption of visible and ultraviolet radiation. Its salting-out properties, and the fact that it contains

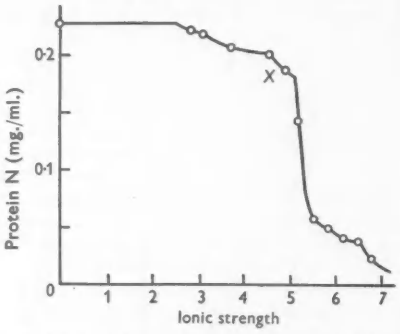

Fig. 2. Precipitation curve of mother liquor from the second crystallization of ox-liver catalase showing the precipitation of a protein *X* in the range of ionic strengths over which a 'photo-precipitation' effect occurs when the solutions are irradiated with light.

nitrogen, and its existence in a solution of a crystallized protein indicate the protein nature of component *X*. Apparently, it is left entirely in the mother liquor after crystallization at pH 6·3, as the precipitation effect was not observed in the solubility test on the twice-crystallized material.

DISCUSSION

The existence of component *A* in the preparation analysed above is undoubtedly due to the action of the Waring Blendor, which disintegrates the tissue much more than mincing. This indicates that *A* is strongly bound to insoluble particles in the liver cells, only being released into solution when these are damaged by the blending process. This explanation is supported by the observation of Falconer & Taylor (1946*a*) that catalase activity is released from thoroughly washed liver mince after autolysis, and by the work of Still & Kaplan (1950), which showed that the action of the Waring Blendor could bring about the transfer of the glycolytic oxidase activity of a suspension of mitochondria from the particulate elements into solution.

The value of 0·43 for the ratio of the optical density at 405 mμ. to that at 275 mμ. for component *A*, indicates that *A* is distinctly different from *C* for which the corresponding ratio is 0·90. These values suggest that either component *A* has a much lower ratio of haematin to protein molecules than *C* or that its protein component has a higher aromatic amino-acid content than the *C* protein. The latter is identical in *Kat.f.* and optical properties with the catalase preparations obtained without the use of the blender and is therefore unbound, or not so firmly bound, to insoluble particles in the cells as the *A* component. In view of the above facts it seems probable that the *C* component is partially degraded erythrocyte catalase, with lowered specific activity due to its content of bile pigment haematin, which is on the pathway of degradation of haematin in liver. This explanation would account for the ease with which it can be extracted and its low activity. The *A* component, however, would appear to be the 'structural' catalase of the liver in view of its high specific activity and the drastic treatment needed for its extraction.

By fractional crystallization of crystalline horse-erythrocyte catalase prepared according to Bonnichsen (1947), Deutsch (1951) has recently obtained preparations with *Kat.f.* values ranging from 84000 to 118000, but with identical absorption properties. The high-activity fraction was found to be extremely labile, the *Kat.f.* value decreasing to 84000 on standing at 0° or on dilution, without any change in its absorption properties. The two components observed in the ox-liver catalase preparation described in this paper have different optical properties, and the highly active component appears in the preparation only after homogenization. This would seem to preclude any relationship between them such as that observed by Deutsch in the case of erythrocyte catalase preparations.

SUMMARY

1. A modification of the Sumner & Dounce method of preparing crystalline ox-liver catalase has been developed which yields a product with a *Kat.f.* of 72000.

2. It has been demonstrated by a solubility test technique that the product contains two distinct catalases with *Kat.f.* values of 45000 and 180000.

3. It is suggested that the catalase with the *Kat.f.* value of 180000 is attached to insoluble complexes in the liver cell and is the structural catalase of liver.

4. A protein exhibiting a reversible change in solubility on exposure to radiation of wavelengths in the visible and ultraviolet regions of the spectrum has been detected in partially purified catalase preparations.

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Effects of Cold Environment on Deposition of Fat in the Liver in Choline Deficiency

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In a preliminary communication (Sellers & You, 1949) it was reported that when rats were exposed to a cold environment the deposition of excess fat in the liver produced by feeding a hypolipotropic diet was effectively prevented. Only one set of experimental conditions had been tested, so that it was of interest to extend the investigation by varying the length of exposure and the type of diet offered, and to study the effects of acclimatization in relation to the phenomenon. Our interest in the effects of the thyroid gland on deposition of fat in the liver (Sellers & You, 1951) also made it desirable to assess the role of this gland in the 'pseudolipotropic' action of cold.

EXPERIMENTAL

In all experiments rats of the Wistar strain, bred in the department, were kept in individual metal cages and were given water *ad lib.* After the experimental period they were killed by cervical dislocation and the livers were removed for chemical and histological examination. Total liver lipids were estimated using the method of Best, Lucas, Patterson & Ridout (1946). The basal diet consisted of peanut meal 30%, casein 6%, salts 4% (Beveridge & Lucas, 1945), sucrose 39%, 'vitamin powder' 1%, beef fat 15%, corn oil 5%, α-tocopherol acetate 0·010%, cod-liver oil concentrate 0·015%. The 'vitamin powder' was of such a composition that the intake per 10 g. of diet was as follows: Biotin 3 μg., thiamine hydrochloride 50 μg., riboflavin 25 μg., pyridoxine hydrochloride 20 μg., calcium pantothenate 100 μg., nicotinic acid 100 μg., folic acid 5 μg., 2-methyl-1:4-naphthoquinone 10 μg., inositol 5 mg., *p*-aminobenzoic acid 1 mg. The cod-liver oil concentrate supplied at least 300 i.u. vitamin A and at least 75 i.u. vitamin D/10 g. of diet. In the high-fat diet the content of fat was increased by adding an additional 20% of beef dripping and 10% of corn oil at the expense of sucrose to make the total content of fat 50%.

Effect of cold environment on acute choline deficiency

(1) Twenty male rats weighing from 170 to 220 g. were divided into two groups and were fed the basal hypolipotropic diet *ad lib.* for 14 days. During the experiment, one group was placed in a cold room maintained at a temperature of 1·5 ± 1°, while the other was kept in the usual animal quarters at a temperature of 22 ± 2°.

(2) Ten adult female rats (170–216 g.), which had been kept in the cold room for 54 days, were fed the hypolipotropic diet instead of their usual ration for a period of 2 weeks. At the same time ten female rats of similar weight range which had lived at 'room temperature' in the animal colony were exposed to cold and were fed the same hypolipotropic diet for a period of 2 weeks.

(3) The thyroid glands of ten adult female rats were removed surgically and the animals were given subcutaneous injections of 5 μg. DL-thyroxine (Roche-Organon) daily for 5 days before and during exposure to cold. While in the cold room the animals were fed the basal hypolipotropic diet *ad lib.* The purpose of this procedure was to obviate the increased production of thyroid hormone which may occur after exposure to cold, and also to make possible the survival

of such animals in the cold environment (Sellers & You, 1950). Ten normal female rats were also kept in the cold room and received daily the average amount of basal diet consumed by the thyroidectomized rats. In both groups body weights ranged from 180 to 200 g. at the commencement of cold exposure.

(4) A hypolipotropic diet of high (50%) fat content was given *ad lib.* to two groups of ten female rats for 14 days. One group was maintained at room temperature, and the other was exposed to cold. A third group of ten females was exposed to cold simultaneously but was fed the basal (moderate fat) diet. The average body weight of all rats was 181 g. when the feeding was started.

The first part of this experiment was repeated using thirty female rats of approximately the same weight range. All rats received the same high-fat, low-choline diet *ad lib.*, but eighteen animals were kept in the cold room during the experimental period, while the remaining twelve lived at normal room temperature.

(5) A group of twenty female rats with body weights ranging from 162 to 190 g. were placed in the cold room and were fed the hypolipotropic diet *ad lib.* At the end of each week two animals were sacrificed, the last four rats being killed at the end of 10 weeks' exposure. The liver of each animal was removed and examined histologically. Other animals fed the same diet were sacrificed after a 15-week period of exposure to cold.

RESULTS

The excessive deposition of fat found in the livers of rats fed the hypolipotropic diet at room temperature did not occur in rats which were given the same diet but were kept in the cold environment. This preventive effect was also observed in rats which had previously been acclimatized to the cold environment. Similar results were obtained with thyroidectomized rats which received a constant daily maintenance dosage of thyroxine. The results are summarized in Table 1. Even after 10 and 15 weeks of exposure the fat content of the livers of rats fed the hypolipotropic diet remained low, in some cases within the normal range (Pl. 3).

When the content of fat in the basal diet was increased to 50%, the total lipid content of the livers was found to be higher than that of the group receiving the basal hypolipotropic diet in the cold environment. However, it was lower than the average figure obtained in the groups of rats which were fed the high-fat diet and were kept at room temperature (Table 2, *A* and *B*). The food intake of all rats exposed to cold was considerably higher than

Table 1. *Effect of cold environment* (2°) *on acute choline deficiency* (14 *days*)

No. of rats	Sex	Group	Environment	Average body wt. (g.) Initial	Average body wt. (g.) Final	Average food intake per day (g.)	Average liver wt. (g.)	Average total lipids (% wet liver wt.) ±S.D.
10	M.	Normal	Cold	195	214	22	12·4	7·2±1·2
10	M.	Normal	Normal	196	248	15	13·8	24·8±4·9
10	F.	Acclimatized*	Cold	191	195	19	8·1	7·20‡
8	F.	Normal	Cold	185	184	17	9·1	7·50‡
6	F.	Thyroidectomized†	Cold	189	182	16	8·3	6·75‡
6	F.	Normal	Cold	186	180	16	9·7	6·70‡

* Rats were in cold environment for 54 days before commencement of low choline diet.
† Each rat received 5γ thyroxine per day.
‡ Livers were pooled for fat determination.

Table 2. *Effect of cold environment on fat deposition in the liver with high-fat, choline-deficient diet* (14 *days*)

No. of rats (female)	Diet	Environment	Average body wt. (g.) Initial	Average body wt. (g.) Final	Average food intake per day (g.)	Average liver wt. (g.)	Average total lipids (% wet liver wt.) ±S.D.
			A				
9	Basal	Cold	183	181	16·3	10·7	8·6*
9	High fat†	Cold	179	180	11·5	10·2	19·9± 9·7
8	High fat†	Normal	181	200	8·9	13·0	23·8±12·2 ($P>0·10$)
			B				
14	High fat†	Cold	168	170	11·76	10·4	18·1± 8·1
12	High fat†	Normal	177	205	10·9	12·2	28·5±12·0 ($P<0·02$)

* Livers were pooled for fat determination.
† 50% fat in basal diet.

that of controls, but the rate of growth as measured by increase in body weight was lower than that of the control rats living at room temperature. The increased consumption of food was more marked in the diets with lower content of fat, and therefore with lesser caloric value.

DISCUSSION

When rats are fed diets low in choline and its precursors, the amount of excess lipid deposited in the liver may be influenced by a variety of factors. Some of these (e.g. intake of food, growth rate, age, sex, previous nutritional status, the presence or absence of other dietary constituents) have been appreciated for many years, others (e.g. environmental temperature, hormonal action) have received less attention. In the experiments reported in this paper the effect of lowering the environmental temperature has been studied, and it is apparent that a definite 'pseudolipotropic effect' due to cold took place. This effect was quite obvious when a diet of moderate fat content (20 %) was fed, but was less so with a diet of high (50 %) fat content. It is of interest to consider whether these results may be explained on the basis of one of the mechanisms described previously or whether some new explanation is necessary.

The energy requirement of a rat exposed to a cold temperature rises greatly, and in our experience the caloric intake is always increased. The rate of growth is adversely affected, but some increase in body weight still takes place. Rats of the range of weight studied have been observed in an environment of 1·5° for as long as a year and a half. In such circumstances it would be unreasonable to claim that the animals were failing to meet their energy requirements from dietary sources. At normal room temperature (22 ± 2°) the basal hypolipotropic diet described here must be restricted greatly in order to achieve the same lowering of liver lipids, and a failure to gain, or a fall in body weight occurs before excessive deposition of fat in the liver is decreased significantly (authors' unpublished data). From this it is concluded that a decrease in the ratio of caloric intake to energy and growth requirements (inanition) cannot alone explain the result.

The preventive action of the cold environment on deposition of fat in the liver is one of degree, however, for when the high-fat diet was fed, large amounts of lipid material were laid down. The average amount of high-fat (50 %) diet consumed was considerably less than that of the basal (20 % fat) diet. Because of the greater caloric values of the high-fat diet, this would be expected.

In studying the endocrine system, exposure to a cold temperature has been used frequently to elicit hormonal responses to conditions of 'stress'. Some of the reactions occur rapidly and are associated with chemical and histological changes in the adrenal cortex. If the pseudolipotropic action of cold were concerned with this type of response, one might expect that the action would be transient in nature. After rats had been fed the basal hypolipotropic diet in the cold for 10 and 15 weeks, the fat content of the liver remained low. In 1950, Sellers, You, Ridout & Best observed that small daily doses (1–2 mg.) of cortisone given to normal rats fed a hypolipotropic diet failed to prevent the deposition of fat in the liver. Within a limited dosage schedule, this finding also applies to cortisone given orally, to deoxycorticosterone acetate and to adrenocorticotropic hormone (ACTH) given by injection (Sellers, You, Ridout & Best, 1951). Thus it appears unlikely that adrenal stimulation is a principal cause of the effect.

A high dose of thyroid substance (0·8 % in the food) is necessary in order to produce a level of lipid approaching the normal, when this basal hypolipotropic diet is fed to rats. Even with such a high dosage, large, centrolobular extracellular fatty cysts are formed (Sellers & You, 1951), a feature seldom if ever seen in the livers of the animals exposed to cold. Therefore, the results obtained do not support the hypothesis that the thyroid plays an important part in the pseudolipotropic effect observed.

Acclimatization does not appear to alter appreciably the pseudolipotropic effect of cold. Acclimatized animals consumed considerably more food than did controls kept at room temperature, yet on chemical examination had significantly lower levels of fat in the livers.

No adequate explanation of the phenomenon can be advanced at present, yet an analysis of energy balance factors affords a basis for future work. The animal in the cold consumes a greater quantity of food than does the control kept at room temperature but does not grow so rapidly. In order to maintain body temperature in the cold, the energy expenditure is much greater and therefore it is logical to suggest that the greater caloric intake is used in producing heat. Very little dietary choline is available, so the necessary metabolic processes must be carried out (1) without choline, (2) by synthesis of choline within the body, or (3) the limited amount of choline present must be used more efficiently. Whichever of these alternatives is correct, it would appear that exposure to cold has brought about an alteration in normal metabolic pathways, and efforts are being made to investigate the possibilities more fully.

SUMMARY

1. When a hypolipotropic diet of moderate fat content (20 %) is fed to rats exposed to a temperature of 1·5 ± 1°, excessive deposition of fat in the liver is effectively prevented.

2. This 'pseudolipotropic' action is a matter of degree, however, for with a high-fat (50%), low-choline diet lipids accumulate in the liver. The fat content of the liver is significantly less than in control animals kept at room temperature on the same type of diet.

3. The pseudolipotropic action of cold is demonstrable in acclimatized as well as in normal animals, and is apparently not mediated to a recognizable extent through either the thyroid or the adrenal gland.

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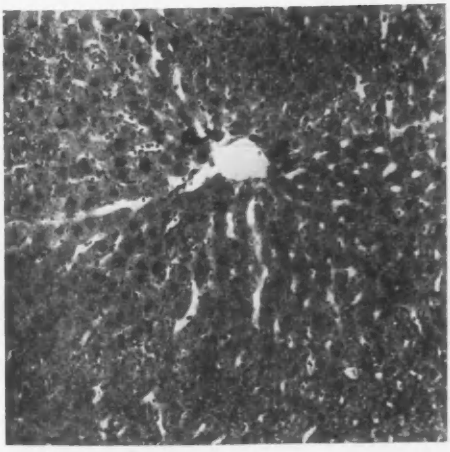
EXPLANATION OF PLATE 3

(*a*) Livers from (left) a rat fed the hypolipotropic diet for 8 weeks in an environment of 1·5°, and (right) from a rat fed the same diet for the same period at room temperature. The liver from the control animal is yellowish in colour and is much larger than that of the rat kept in the cold.

(*b*) Photomicrograph of liver tissue (Orange red O × 135) from a rat fed a hypolipotropic diet for 8 weeks in an environment of 1·5°. The appearance of the section is essentially normal.

(*c*) Photomicrograph of liver from rat fed the same diet but kept at room temperature. Note the globules of fat deposited throughout the lobule.

(*a*)



(*b*)

(*c*)

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